Immunotherapy for Nonmelanoma Skin Carcinoma

Does It Have a Future?

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BACKGROUND. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin are the most common malignancies in the white human population, accounting for greater than 95% of nonmelanoma skin carcinomas (NMSCs). Current data show an increasing incidence of NMSC in recent decades. Although the mortality is low, this cancer group is associated with substantial morbidity. Multiple treatment modalities are available for NMSC, with surgery being a "cornerstone" of current therapy approaches. However, in patients with multiple lesions or in cases of tumors on critical locations, disfigurement and the disease recurrence may represent a serious problem associated with the surgical treatment. The purpose of this study was to review and analyze whether NMSC could represent targets for immune therapy, evaluating the aspects of the availability of tumor antigens and the existence of tumor specific immune response, including a summary of the major clinical studies dealing with immunotherapy for NMSC.

METHODS. The authors have reviewed the available medical literature on NMSC, with a focus on tumor immunology and associated abnormalities, as well as immunotherapy-based treatment trials.

RESULTS. The major advantage of NMSCs is that they arise from the skin, which makes them easily detectable and treatable. Furthermore, these tumors posses all the prerequisites, i.e., the presence of tumor-associated antigens as well as the tumor specific immune response, needed for immune intervention. This also was confirmed in various studies demonstrating clinical efficacy of cytokines, immune response modifiers, or gene therapy in NMSC. AQ: 1

CONCLUSIONS. In addition to clinical cure, by activating and stimulating patient's immune resources this therapeutic option may be a "silver bullet," providing a long-term protective immunity against initial tumor. *Cancer* 2002;94:000–000. © 2001 American Cancer Society.

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Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin are the most common malignancies in the white human population[1,2] These tumors account for greater than 95% of nonmelanoma skin carcinomas (NMSCs) and are associated with substantial morbidity. In men, they are more frequent than prostate carcinoma, and in women they outnumber the breast carcinoma.[1,3] Fortunately, the mortality from these cancers is conversely low.[1] Available data suggest an increasing incidence of NMSC worldwide in recent decades.[1,2]

That they arise primarily in skin makes them easily detectable and clinically manageable. Surgical therapy stands still as a "gold standard" reaching up to a 99% cure rate when the histopathologic margins are free of tumor cells.[4,5] However, substantial disfigurement AQ: 2

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TABLE 1
Categories of Human Tumor Antigens Identified in Squamous Cell Carcinomas[a]

Tumor antigen category	Examples
Cancer-testis antigens (expressed in different tumors, but in normal tissue expression is highly restricted to testis)	NY-ESO-1, SCP-1
Antigens derived from mutated gene products (mutations of genes encoding constitutive cellular proteins)	p53
Amplified gene products (overexpression of constitutive cell proteins)	HER-2/*neu*, MUC-1, Ep-CAM

[a] Reviewed in Old and Chen,[10] Knuth et al.,[11] Stockert et al.,[12] Piyathilake et al.[13]

can result because of the nature of the tumors and their frequent localization on highly visible, cosmetic "important" areas. That, in turn, may represent a serious problem in cases of multiple tumors encountered with certain genodermatoses (i.e., xeroderma pigmentosum, epidermodysplasia verruciformis, or nevoid basal cell syndrome) or immunosuppressed states (i.e., transplant recipients, immunodeficiency syndromes).[1] The recurrence rate after conventional treatments can be up to 20% for both BCC and SCC.[1,4] In addition, patients with a history of NMSC have an increased risk to develop a subsequent primary NMSC.[1]

DO NONMELANOMA SKIN CARCINOMAS FULFILL THE PREREQUISITES FOR IMMUNOTHERAPY?

Among epithelial tumors, NMSCs are unique with respect to their biologic behavior. They grow slowly and rarely metastasize. Moreover, BCC and SCC have the potential to regress spontaneously, with BCC showing histologic evidence of partial regression in 50% of the cases.[6–9] All this is implicative of existence of a specific antitumor response partially controlling the tumor progression, which might have a considerable therapeutic impact when understood and exploited. The approach that should warrant success both in local (complete clearance, no recurrence) and systemic aspects of tumor control (induction of "protective" immunologic memory) needs to have three major conditions fulfilled: 1) the expression of tumor specific antigens, 2) intact antigen processing machinery and proper presentation of tumor antigens through histocompatibility leukocyte antigen (HLA) molecules, and 3) existence of T cells recognizing these antigens.

T1 In recent years, a whole series of tumor-associated antigens have been identified in SCCs (Table 1). Many of these molecules initially were identified by the serologic analysis of recombinant cDNA expression libraries (SEREX),[14] an approach that permits the identification of tumor antigens eliciting immunoglobulin G antibody responses in the autologous tumor patient. In theory, antigens uncovered by SEREX were not supposed to be recognized by cytotoxic T lymphocytes (CTL). However, subsequent studies demonstrated that some of them, i.e., NY-ESO-1, could be the targets for CTLs as well.[15] In NMSC, mutated p53 is found in greater than 90% of SCC and in most BCC.[16] The nature of this mutation represents a classic "fingerprint" for the effect of ultraviolet radiation (UVR) on DNA,[17] supporting to the role of UVR in the onset of skin carcinoma.

Various alterations affecting expression of HLA class I antigens have been demonstrated in cutaneous BCC and SCC, such as the loss of HLA-A, -B monomorphic determinants, the down-regulation of total surface class I expression, and the differential heavy chain and β_2-microglobulin expression.[18–22] However, the expression of HLA class I antigens seemed to be more heterogeneous throughout the lesion rather than completely absent (only in < 10% of cases),[18–21] which, in turn, suggests the existence of tumor cells capable of presenting tumor peptides to T cells.

The tumor nests of BCC and SCC frequently are surrounded by a varying degree of mononuclear cells, suggestive that these tumors are immunogenic because the host has initiated an immune response potentially controlling their growth.[8,9,23] The infiltrate consists mainly of T cells with a relative paucity or absence of B lymphocytes and natural killer (NK) cells.[8,23] These T cells are only sporadically observed infiltrating the tumor mass, with CD8+ T cells dominating the total CD3+ T-cell population (CD4 to CD8 ratio between 2 to 4).[8,24,25] Hunt et al. found a fivefold increase in T-cell number in actively regressing BCC suggesting their central role in tumor regression.[26] A study by Haeffner et al. showed that the tumor-infiltrating lymphocytes (TILs) in SCC are polyclonal and major histocompatibility complex (MHC)–restricted CTLs expressing the T-cell receptor $\alpha\beta$ heterodimer.[8] These TILs expressed HLA-DR (indicative of a state of activation) and in the in vitro system efficiently lysed autologous SCC cells. Although TILs in BCC and SCC frequently express some "activation" markers (e.g., interleukin [IL]-2 receptor,[26] HLA-DR,[8] perforin[25]), in vivo lysis of tumor cells fails to happen. One of the explanations may be in the finding that tumor cells in BCC and SCC often produce various immunosuppressive Th2 type cytokines,[20,27] which results in inhibition of cell-mediated immune response, depletion of the HLA-DR and CD1a positive antigen-presenting cells (APC) in the skin, down-regulation of costimulatory

CD80 and CD86 molecules on APC, thus abrogating immune recognition as shown by Nestle et al.[28]

It appears that there are many missing links in this chain leading to establishment of an effective tumor specific immune response. Actually the abnormalities described above were the rationale for the immune intervention at different levels in NMSC.

CURRENT TREATMENT APPROACHES

Interferons

Interferons (IFNs) are a group of naturally occurring glycoproteins exerting multiple biologic effects, such as the control of cell growth and differentiation, regulation of cell surface antigen expression, and modulation of humoral and cellular immunity.[29,30] Results T2 from available clinical trials (summarized in Table 2) indicated that interferon can be an effective treatment for BCC and SCC. In the series by Cornell et al.[31] and Chimenti et al.,[32] multiple intralesional injections of IFN-α2 induced overall remission rates of 70– 100% in BCC patients (Table 2). Increased numbers of CD4+ T cells infiltrating the dermis and surrounding the BCC nests have been observed after intralesional injections of IFN-α.[33] The mechanism of IFN-α action in tumor regression involves a shift toward secretion of Th1 type cytokines, i.e., IFN-γ and IL-2[30] facilitating cellular immunity, up-regulation of CD80 and CD86 costimulatory molecules on APC, and enhancement of HLA class I and class II expression,[20] or induction of apoptosis via CD95 up-modulation on BCC cells interacting with CD95 receptor on infiltrating CD4+ cells.[33] Five- and 10-year follow-up period in the series of Ikic et al. showed no recurrences in BCC patients with complete response to intralesional IFN-α treatment[34] (Table 2).

Using IFN-β, remission rates between 50% and 100% were reported (Table 2), with an advantage in that the total dose inducing complete remission in BCC was lower for IFN-β than IFN-α, and that the side effects were less frequent.[35] Available data on the use of IFN-γ in BCC treatment are somewhat contradictory, with no cure rate above 50% (Table 2) and frequent side effects.[36,37] Of note is that the tumor type and size have to be considered before the therapy, because of the lower efficacy (40%) in treating morpheaform BCC.[30]

Squamous cell carcinoma has been reported to respond to intralesional IFN-α treatment, with a response rate up to 98% (summarized in Table 2). Ikic et AQ: 3 al. treated 52 patients with SCC, of which 32 in early eighties. After one decade of observation, two recurrences at the site of original lesion occurred, suggesting a possibility for persistent cure in SCC patients treated with IFN.[34]

Interleukin-2

Interleukin-2 is a cytokine secreted by CD4+ T lymphocytes after antigen recognition. It has no direct effect on cancer cells, in contrast with IFN-α. Its antitumor activity is achieved through immunomodulation by stimulating cytotoxicity of T lymphocytes, NK cells, and macrophages.[29] Intralesional IL-2 has been shown to be beneficial in the treatment of various epithelial carcinomas including BCC, Bowen disease, and metastatic eccrine poroma.[38,39] We have successfully treated multiple BCC in a patient with nevoid basal cell syndrome by daily intralesional injections of 0.5 mL recombinant IL-2 (Proleukin, Roche) divided AQ: 4 on 3 lesions, for 5 days. However, the treatment induced fever, flu-like symptoms, and pain at the injection site. Kaplan and Moy conducted a study treating 12 BCCs with perilesional injections of polyethylene glycol (PEG)–IL-2 to minimize the systemic toxicity.[40] AQ: 5 The cure rate was 66.6%, and the treatment was well tolerated, except for 1 patient who developed systemic side effects.

It is evident that intralesional application of cytokines is beneficial in NMSC. However, the application of these substances can have notable disadvantages including the side effects (local pain, skin necroses, and flu-like systemic symptoms) and a very short half-life necessitating repeated injections. Especially if several lesions are treated simultaneously and the total dose reaches a certain maximum (e.g., more than 3 million IU of IFN-α per day), the treatment often is associated with severe systemic side effects. A different method of preparation (e.g., PEG-IL-2) might be one of the solutions, providing longer half-life and reduced immunogenicity, reducing the number of daily applications and enabling the treatment of multiple lesions.

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Imiquimod: A Potent Local Immune Response Modifier

Immune response modifiers or immunomodulators are substances that directly influence a specific immune function or modify one or more components of the immunoregulatory network to achieve an indirect effect on specific immune function.[5] Imiquimod, 1-(2-methylpropyl)-1H-imidazo-[4,5-c]quinolin-4-amine, is one such agent with the potent antiviral and antitumor activity in animal models.[41] It has been approved recently for the topical treatment of external genital warts. The application of imiquimod induces a whole cascade of cytokines with profound effects on innate and acquired immunity. The activity of this drug results primarily from the IFN-α induction.[41,42] Furthermore, imiquimod stimulates innate immune response through release of tumor necrosis factor-α

TABLE 2
Clinical Efficacy of Interferons in BCC and SCC

	Reference	No. of patients	Objective response rate (%)	Complete response (%)	Partial response (%)	Dosage	Follow-up
Basal cell carcinoma							
IFN-α	Edwards et al. 1990[71]	65	80/[a]52	—[a]		i.l. sustained-release IFN-α-2b formulation 10 million IU/injection once weekly for 3 weeks/[a] or only once in 3 weeks	
	Cornell et al. 1990[31]	172	86			i.l. IFN-α-2b 1.5 million IU 3 times weekly for 3 weeks	1 year, 81% tumor free
	Ikic et al. 1991[72]	86		70.9	15.12	Natural leukocyte IFN	
		20		70	30	IFN alpha-2c	
	Ikic et al. 1991[73]	72		72.2	12.5	Natural leukocyte IFN 400,000–1.2 million IU/weekly for 3–6 weeks	Summarized in the report from 1995
		12		41.7	58.3	IFN-α-2c 2–5 million IU 5 times weekly for 4 weeks	
	Ikic et al. 1995[34]	110		68.2		Natural leukocyte IFN i.l. 300,000–1.2 million IU 12–35 times total in 3–6 weeks	5 years, 43 cases with CR, no recurrences observed; and 10 years 32 cases with CR, no recurrences observed
		20				IFN-α-2c 2–5 million IU/daily (except weekends) 20 times total in 4 weeks	3–5 years, no recurrences observed in CR and PR cases
	Chimenti et al. 1995[32]	140		67.1	23.6	i.l. and perilesional 1.5–3 million IU 3 times weekly for 4–8 weeks	median 36 months, no recurrences observed
	Alpsoy et al. 1996[74]	15		66.6	33.3	i.l. IFN-α-2a 3 times weekly total 10 injections	6–24 months, no recurrences observed
		15		66.6	26.6	i.l. IFN-α-2b 3 times weekly total 10 injections	
		15		73.3	26.6	i.l. IFN-α-2a and -2b 5 times each on alternative regimen	
IFN-β	Kowalzick et al. 1994[35]	87		50		i.l. rIFN-β 3 million IU 2 times weekly for 3 weeks	
				64–86 (dose dependent)		i.l. rIFN-β 0.5–1 million IU 3 times weekly for 3 weeks	
IFN-γ	Edwards et al. 1990[36]	15		7		i.l. IFN-γ 20,000 IU 3 times weekly for 3 weeks	
		14		50		i.l. IFN-γ 100,000 IU 3 times weekly for 3 weeks	
Squamous cell carcinoma							
IFN-α	Ikic et al. 1991[75]	32		59.4	31.2	Human natural leukocyte IFN 400,000–5 million IU weekly for 3–6 weeks	
		10		40	50	IFN-α-2c 5 times weekly for 4 weeks	Summarized in the report from 1995
	Ikic et al. 1991[72]	45		64.4	28.9	Natural leukocyte IFN	
		10		40	50	IFN-α-2c	
	Edwards et al. 1992[76]	36	88.2	97.1		i.l. IFN-α-2b 1.5 million IU 3 times weekly for 3 weeks	
	Ikic et al. 1994 (lower lip)[77]	15	81	48		Natural leukocyte IFN	
		6	67	17		IFN-α-2c	
	Ikic et al. 1995[34]	52		59.6		Natural leukocyte IFN 400,000–1.2 million IU total in 12–30 injections	More than 5 years, 19 patients followed, 2 recurrences
		10				IFN-α-2c 2–5 million IU/daily (except weekends) 20 times total in 4 weeks	3–7 years, no recurrence observed

[a] Blank fields state that the parameter of interest was not reported in the article.

i.l.: intralesional; rIFN: recombinant IFN; CR: complete response; PR: partial response.

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(TNF-α) and IL-6, leading to enhancement of NK cell activity and activation of macrophages to secrete cytokines and nitric oxide and inducing proliferation and differentiation of B lymphocytes.[41,43] In addition to effects mediated through IFN-γ, imiquimod promotes shift toward Th1 or cell-mediated immune response through induction of IL-12, IFN-γ, and activation of CTLs.[44] Imiquimod-induced IL-12, TNF-α, and IFN-α may have potential antiangiogenic effects.[45] After application of imiquimod, the number of Langerhans cells in the epidermis decreases with concomitant change in their morphology, suggesting their activation by imiquimod.[46] Moreover, imiquimod seems to enhance the migration of Langerhans cells to draining lymph nodes leading to enhanced antigen presentation.[46] From different aspects, imiquimod help foster a stronger cell-mediated immune response, which is important in control and long-term protection from the initial virus or the tumor.

Because BCC is known to respond to IFN, Beutner et al. performed a randomized, double-blind pilot trial to evaluate the efficacy and safety of topical 5% imiquimod cream versus vehicle in the treatment of BCC.[47] Twenty-four patients with primary superficial or nodular BCC received imiquimod 5% cream in 1 of 5 dosing regimens for up to 16 weeks. Complete responses were observed in 100% of patients with once-daily, twice-daily, or thrice-weekly regimens. Complete responses also were observed in 60% patients with the twice-weekly regimen and in 50% of those who received once-weekly treatment. In all imiquimod groups, 83% of patients responded (determined histologically as complete clearance of the lesion) irrespective of dosage schedule.[47] Imiquimod has been shown to have an acceptable safety profile with tolerable side effects, which were dose-dependent (mild-to-moderate erythematous, flaking and edema at the lesion site, burning at the application site, and pain in less then 10% of patients).[47]

The proposed mechanism for imiquimod action in BCC is the reversal of immunosuppression caused by the chronic sun exposure involved in the onset of skin carcinoma. Ultraviolet radiation damages keratinocytes that then secrete IL-10, TNF-α, and isomerized urocanic acid, impairing the function of APC (Langerhans) cells and favoring the development of Th2 cytokine microenvironment, which then suppresses antitumor T cells.[5,47] The principal role of effective immunosurveillance in BCC is substantiated by the finding that 1) UVR has immunosuppressive activity, 2) BCC incidence is increased in renal allograft recipients, 3) BCCs respond to IFN therapy, as AQ: 6 demonstrated by imiquimod.[47] Of note, imiquimod up-regulates the same cytokines that are down-regulated in BCC, leading to immunologic restitution of sun-damaged skin and elimination of malignant cells.[5,47]

Imiquimod-induced Th1 cytokine switching together with increased antigen presentation may be of benefit in patients with underlying immune disorders, such as chronic lymphocytic leukemia (CLL) and human immunodeficiency virus (HIV)–1 disease, known to induce Th2 cytokines.[48,49] In five patients with CLL, trice-weekly imiquimod treatment in combination with COX-inhibitor sulindac (additionally potentiating Th1 cytokine pattern), induced clinical resolution and histologic clearance of the Bowen disease (SCC in situ).[48] Pehoushek and Smith have treated in situ anal and perianal human papillomavirus positive SCC in an HIV-1 positive man with imiquimod and 5% fluorouracil.[50] After thrice-weekly imiquimod regimen combined with daily application of 5% fluorouracil, clinical as well as mucosal cytologic and histologic examination showed no evidence of residual dysplasia. Apart from efficacy and safety in the treatment of anogenital warts in HIV-infected individuals,[49] imiquimod might represent a reasonable nonsurgical alternative for the management of HIV-related neoplasia, i.e., basal and SCCs.

The removal of multiple tumors associated with certain genetic conditions often produces discomfort caused by the surgical procedure itself and the tumor load. Imiquimod has been applied with success in the treatment of multiple tumors associated with basal cell nevus syndrome.[51] In a patient having xeroderma pigmentosum, we have treated successfully multiple BCC with imiquimod applied daily for 3 weeks (Fig. 1). F1 The complete clinical remission was accomplished and confirmed histologically.

Imiquimod treatment of tumors localized on cosmetically difficult parts (e.g., scalp, shin) seems promising.[52,53] In a Phase II, open-label study, Mackenzie-Wood et al. have treated 16 patients with Bowen disease with once-daily imiquimod application for 16 weeks.[53] Complete clinical response with histologic clearance was achieved in 93% of the patients treated.[53]

Gene Therapy

Somatic gene therapy is a new treatment option that introduces genes into cells not involved in reproduction. Skin rapidly becomes a major organ for genetic manipulations because of its accessibility (i.e., direct injection, gene gun) and immunologic properties (numerous professional APC, epidermal-Langerhans or dermal dendritic cells involved in the induction of long-lived T-cell–mediated immunity). This, in turn, opens a broad range of completely new treatment modalities for skin carcinoma, some of which have

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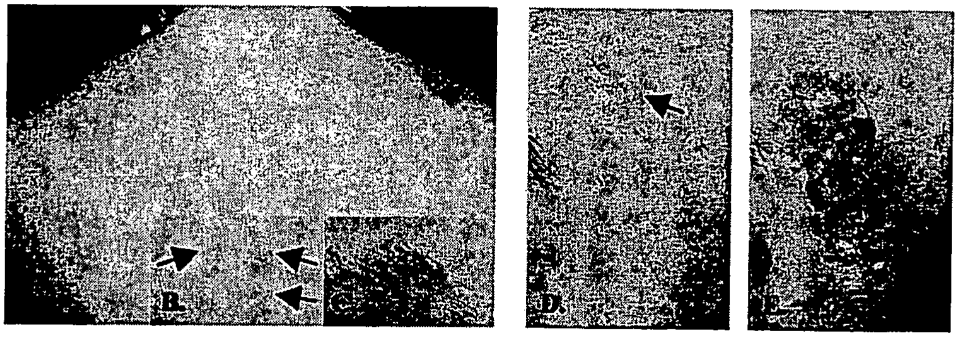

FIGURE 1. Basal cell carcinomas on the shoulder (B) and glabellar area (D) before imiquimod treatment in a patient with xeroderma pigmentosum (autosomal recessive disorder with defective DNA repair machinery that results in susceptibility to ultraviolet radiation–induced cancer, i.e., basal cell carcinoma and squamous cell carcinoma); the ulceration followed by the clearance of the tumor after imiquimod treatment is also shown (A, C, E).

been applied already with success in melanoma.[54–57] The most frequently used delivered genes have been those designated to kill tumor cells directly, such as suicide genes, or to induce immune-mediated destruction, such as immunogenic antigens and cytokines.[54,57,58]

Additional options in turning the aberrant biology of the tumor against itself include targeting genetic mutations specific to tumor cells or delivering the genes that stands as a sentinel over control of cell cycle, such as p53.[58] p53 mutations are common in AQ: 7 NMSC, and even one mutated p53 gene may be sufficient to cause loss of tumor suppressing activity.[59] The importance of intact p53 is clearly demonstrated in p53 knockout mice, which show a marked increase in rate of skin carcinoma development after UVR exposure.[59] The restoration of the p53 status in NMSC might be an imperative, making them perfect targets for gene therapy. We recently have conducted a Phase I escalation study of single intratumor injection of replication-defective adenoviral expression vector containing the wild-type p53 gene in patients with metastatic melanoma and breast carcinoma.[60] Pretreatment lesion biopsies demonstrated increased p53 protein immunoreactivity. Gene transfer resulted in the biologic activity of the transgene in tumor tissue, as confirmed by reverse transciption–polymerase chain reaction. Our Phase I trial and the experiences of other groups[61,62] show that the intralesional application of such adenoviral vectors is a safe, feasible, and biologically effective treatment. Another promising approach applied is based on targeting the cells expressing aberrant p53, using ONYX-15 adenovirus that AQ: 8 selectively replicates in and lyse p53-mutated cells.[63,64]

In addition, tumors arising in the skin represent an ideal target for gene delivery leading to local sustained protein expression (i.e., IFNs), minimizing the need for surgery, multiple IFN injections, and associated side effects. Hottiger et al. have compared the effects of liposome-mediated IFN gene transfer with recombinant IFN-α2 injections on human BCC transplanted onto severe combined immunodeficiency mice.[65] Direct injection of DNA encoding IFN-α2 lead to its prolonged local expression in comparison to intralesional IFN-α2 treatment and induced BCC regression.[65]

Interleukin-12, another cytokine that has shown clinical efficacy,[66] may be useful for gene therapy in NMSC. Interleukin-12 has a central role in directing immune response, because it promotes the switch to Th1 pathway while inhibiting Th2 cytokines,[66] known to be expressed in NMSC.[20,27] It enhances the innate immune response, inducing NK lytic activity and IFN-γ secretion.[66] Clinical data from melanoma Phase I study conducted by Sun et al. are encouraging in inducing antitumor response in patients vaccinated with IL-12 gene-modified autologous melanoma cells.[67] Injection of plasmid DNA encoding human IL-12 has resulted in the regression of melanoma metastases in gray horses, demonstrating the effectiveness and safety of this therapeutic approach in a large animal model.[68]

An advanced version of the immunostimulation would be gene delivery of costimulatory molecules that are lacking in BCC but are needed for proper T-cell activation, i.e., ICAM-1, CD80, and CD86.[66,69] The main precaution would be to avoid the binding of CD80/CD86 to CTLA4 that leads to inhibition of T-cell response.[66,69] In a combined approach using melanoma cells transduced with CD80 and IL-12, we have demonstrated a strong proliferative response from peripheral blood mononuclear cells on contact with transduced melanoma cells, at high levels of Th1 cytokine production, increased expression of HLA class I and II antigens, and ICAM-1 on transduced melanoma cells,[70] suggesting its clinical applicability.

PERSPECTIVES

Different treatment options are available for treatment of NMSC: simple excision, Mohs micrographic surgery, curettage and electrodesiccation, cryosurgery and irradiation therapy. All of them are relatively effective, but are associated with pain and scarring, and may be cosmetically deforming. Given the rising incidence and prevalence of NMSC worldwide, it appears reasonable to seek an additional therapeutic modality targeting malignant disease itself, rather then just simply removing it. Particularly in difficult situations related to tumor histologic type, size, number, problematic location, concomitant disease (bleeding diathesis or medications with bleeding tendency) or in cases where surgical and radiologic therapy is rejected or contraindicated. On the other side, immunological and genetic abnormalities associated with NMSC are up to the present time sufficiently elucidated. Yet, the studies employing this knowledge to fight and overcome the problem, are in contrast with its incidence surprisingly rare. Nonmelanoma skin carcinomas posses all the prerequisites to be a suitable subject of immune intervention, which might in turn provide a long-term protective immunity. We are convinced that local immunomodulation represents the future in this field, and hope that this review will contribute to its further development.

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Immunotherapy for Nonmelanoma Skin Carcinoma: Does It Have a Future?

Mirjana Urosevic, and Reinhard Dummer

Basal cell and squamous cell carcinoma of the skin are the most common malignancies in the white human population, accounting for greater than 95% of nonmelanoma skin carcinomas. Nonmelanoma skin carcinomas posses all the prerequisites, i.e., the presence of tumor-associated antigens as well as the tumor specific immune response needed for immune intervention, which was confirmed in various studies demonstrating clinical efficacy of cytokines, and immune response modifiers in treating these cancers.

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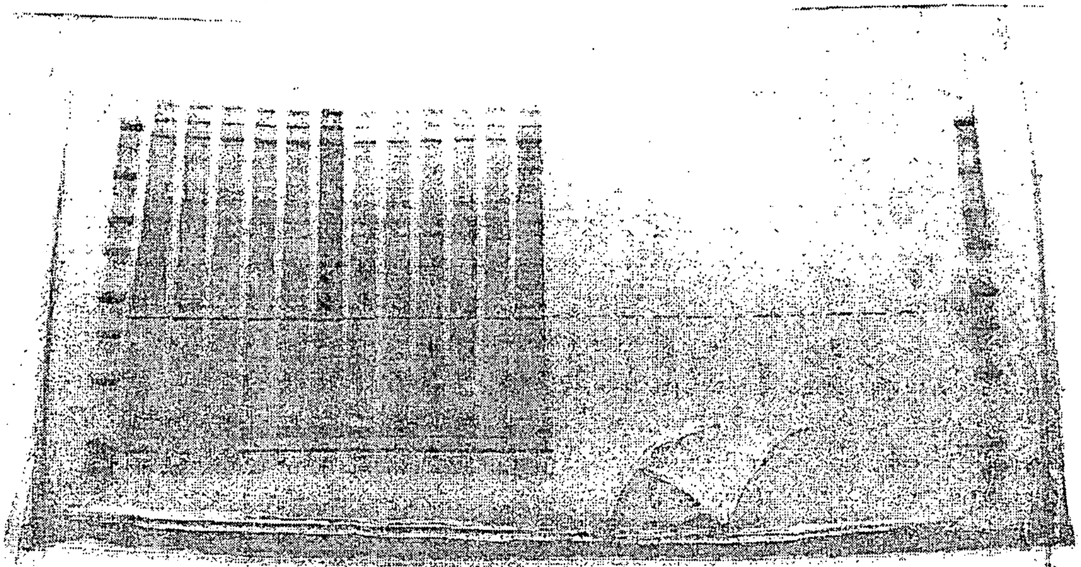

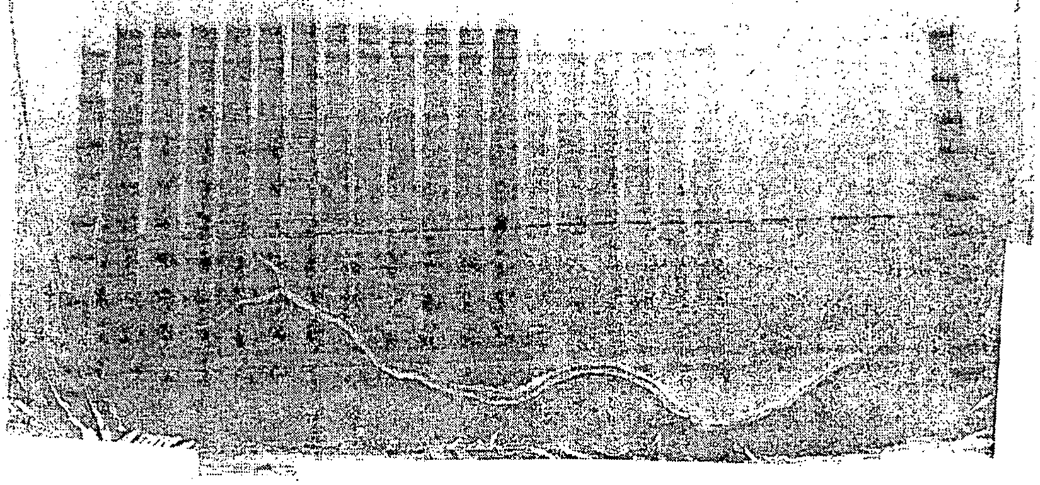

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Clinical and laboratory studies

Treatment of basal cell carcinoma with intralesional interferon

Hubert T. Greenway, M.D.,* Roger C. Cornell, M.D.,* Daniel J. Tanner, M.D.,**
Edwin Peets, Ph.D.,** Gerald M. Bordin, M.D.,*** and
Constance Nagi, M.D.**** *La Jolla, CA, and Kenilworth, NJ*

Eight patients with basal cell carcinomas were treated with recombinant alpha-2 interferon. Each patient had a biopsy-proved basal call carcinoma of the nodular or superficial type that was injected intralesionally three times a week for 3 weeks (9 total injections) with 1.5×10^6 IU (0.15 ml) of alpha-2 interferon per injection (total dose, 13.5×10^6 IU). Excisional biopsy 2 months after completion of therapy revealed no evidence of basal cell carcinoma in any patient. Minimal side effects were observed. In these eight patients alpha-2 interferon was therefore an effective and safe modality of treatment. The encouraging results of this pilot study suggest that additional evaluation of interferon in the treatment of basal cell carcinoma is warranted. (J AM ACAD DERMATOL **15**:437-443, 1986.)

Basal cell carcinomas are the most common cutaneous neoplasms found in man, comprising the majority of the 500,000 new cases of nonmelanoma skin cancer that develop each year. A variety of clinical and histologic forms of basal cell carcinoma exist, including nodular-ulcerative, superficial, pigmented, morphea-like, fibroepitheliomatous, and the nevoid syndrome.[1] Treatment methods include electrodesiccation and curettage, excision, cryosurgery, and irradiation, with high cure rates in the range of 95%.[2] Certain primary basal cell carcinomas and recurrent basal cell carcinomas may respond best to Mohs' surgery; these may present a demanding therapeutic challenge. Cure rates via Mohs' surgery approach 99% for all primary basal cell carcinomas and 96% to 98% for recurrent basal cell carcinomas.[3,4] Despite the high cure rates effected by surgical technics, nonsurgical approaches to therapy may offer advantages. Intralesional treatment with spleen extract in 1951* and bleomycin in 1975 (R. C. C.) have been tried but have been unsuccessful. This study describes our evaluation of the effectiveness of intralesionally injected interferon on basal cell carcinoma.

Interferons are a family of proteins first identified for their antiviral property of interfering with in vitro viral replication.[5] In addition, they also inhibit cell growth, effect cell differentiation, and modulate a variety of other immunologic and cellular functions. Interferons are divided into three types: alpha (α) or leukocyte interferon, beta (β) or fibroblast interferon, and gamma (γ) or immune interferon. Alpha interferon is a naturally occurring mixture identified by recombinant deoxyribonucleic acid (DNA) technics; its production can

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*Mohs, F. E., M.D.: Personal communication, 1985.

Table I. Characteristics of cases

Case	Age	Sex	Type of basal cell carcinoma	Pretreatment lesion size (mm)	Posttreatment lesion size (mm)	Lesion flattening (nodular lesions only)	Follow-up excisional biopsy
1	59	M	Superficial	8 × 8	6 × 3	—	No tumor noted
3	63	M	Superficial	8 × 5	0	—	No tumor noted
5	50	F	Superficial	14 × 12	3 × 2	—	No tumor noted
7	51	M	Superficial	11 × 8	6 × 4	—	No tumor noted
8	58	M	Superficial	12 × 10	6 × 4	—	No tumor noted
2	52	M	Nodular	7 × 6	5 × 4	100	No tumor noted
4	50	F	Nodular	9 × 8	6 × 3	100	No tumor noted
6	48	M	Nodular	11 × 6	6 × 4	100	No tumor noted

be virally induced in peripheral blood leukocytes or cultured lymphoblastoid cells; alpha-2 interferon can be produced in bacteria using recombinant technics.[6] Recombinant alpha-2 interferon has been shown to be safe and effective in clearing single and multiple condylomata (but not plantar warts).[7] Interferons have also been utilized as anticancer agents in a number of studies for such diverse neoplasms as hairy cell leukemia,[8] cutaneous T cell lymphoma,[9] Kaposi's sarcoma,* and malignant melanoma.[10] Results with hairy cell leukemia have been the most impressive.

PATIENTS AND METHODS

Patients

Eight patients (six male and two female) ranging in age from 48 to 63 years (average age, 54 years), each with one biopsy-proved primary basal cell carcinoma of the nodular (3 patients) or superficial (5 patients) type, were included in the study. The locations of the lesions included the back (5), the shoulder (1), the arm (1), and the neck (1). Lesion size varied from 7 × 6 mm to 14 × 12 mm. The diagnosis of nodular or superficial basal cell carcinoma was confirmed by incisional biopsy at least 1 week prior to the initiation of treatment. This incisional biopsy (mainly 2 or 3 mm punches taken from the edge of the lesion) was limited in size to 25% or less of lesion size. Each patient was in good health and the basal cell carcinoma to be injected was considered treatable by conventional modalities. Patients with recurrent lesions, genetic or nevoid conditions, central facial or periorificial lesions, lesions with deep tissue involvement, prior exposure to arsenic or radiation, or with morpheaform or pigmented lesions were excluded from this study. Patients were informed of their diagnosis and other available treatment modalities and chose this mode of treatment. Prior to treatment, each patient underwent a complete history and physical examination. The lesion to be treated was then measured and photographed and detailed as to specific location (i.e., right side of back, 11 cm to the right of midline at T12, etc.). The study was discussed thoroughly and the patients gave informed consent.

Methods

Laboratory tests. Pretreatment laboratory tests included a complete blood count with differential white blood cell count, a chemistry profile including renal and liver function tests, and a urinalysis. A white blood cell count was repeated at 24 hours after the first injection of interferon, with the entire hematology and chemistry panels repeated weekly during the 3 weeks of treatment and 1 week following completion of treatment.

Treatment. Treatments were conducted only at the Scripps Clinic and Research Foundation with the human recombinant alpha-2 interferon (SCH 30500) prepared as freeze-dried material and supplied in vials. Dilution with 1 ml of sterile water (preservative-free) produced an isotonic solution containing the appropriate concentration of interferon; 0.15 ml delivered 1.5×10^6 IU. Each lesion was injected with 0.15 ml of alpha-2 interferon via a 30-gauge needle on a tuberculin syringe. The needle was inserted tangentially into the lesion with care being taken to inject the entire amount intralesionally. The procedure was repeated for a total of three injections per week (Monday, Wednesday, and Friday) for 3 weeks. Each lesion was injected with a total of 13.5×10^6 IU (1.5×10^6 IU × 9 doses).

Patients were evaluated during the treatment both for clinical response and for local and systemic side effects. Evaluations were continued at 1, 4, and 8 weeks after

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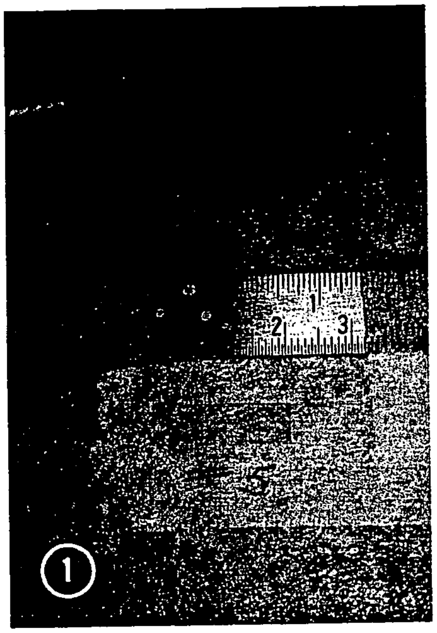
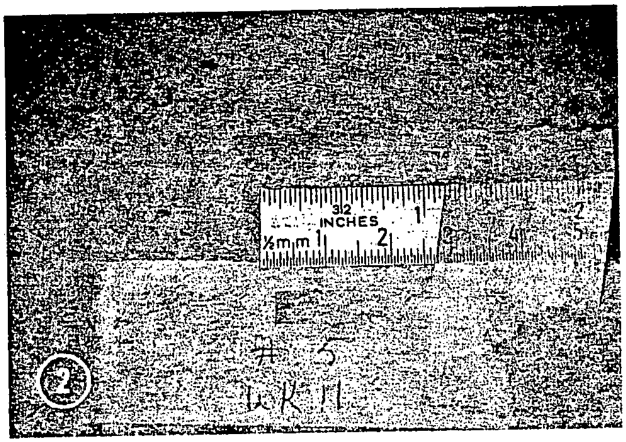
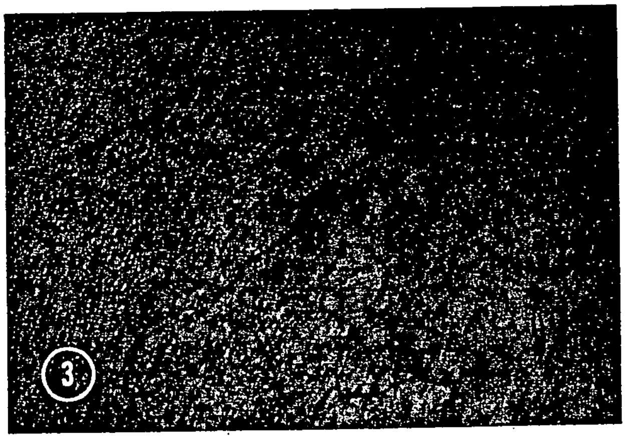
*Rios A, Mansell P, Newell G, et al: The use of lymphoblastoid interferon HV IFN alpha-(Ly) in the treatment of acquired immunodeficiency syndrome (AIDS) related Kaposi's sarcoma (KS). Proc Am Soc Clin Oncol 3:63, 1984. (Abstract C-245.)







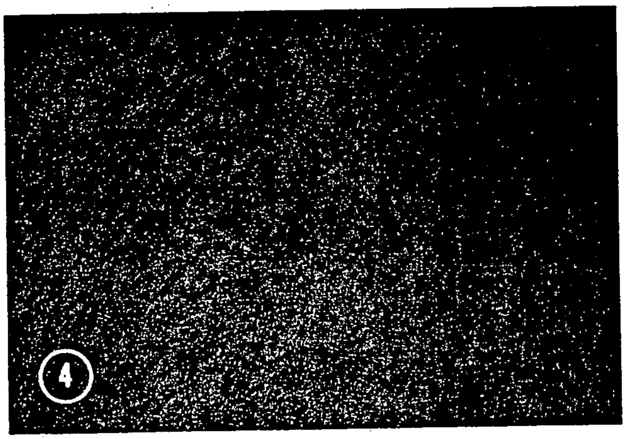


Fig. 1. Case 5. Superficial basal cell carcinoma (pretreatment).

Fig. 2. Case 5. Eight weeks after completion of interferon injections (week 11, day 5) immediately prior to excisional biopsy.

Fig. 3. Case 6. Nodular basal cell carcinoma (left side of back prior to treatment).

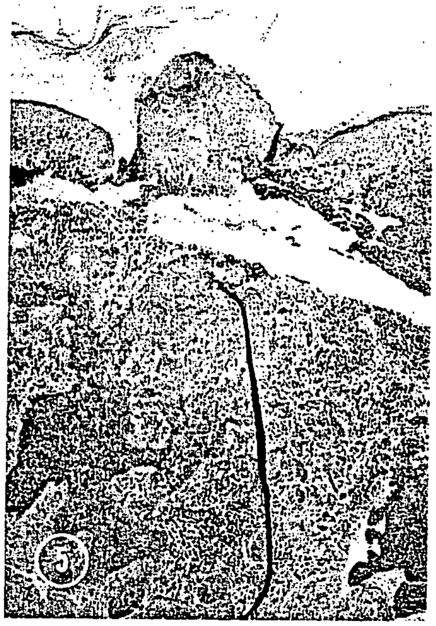
Fig. 4. Case 6. Eight weeks following completion of treatment (immediately prior to excisional biopsy).

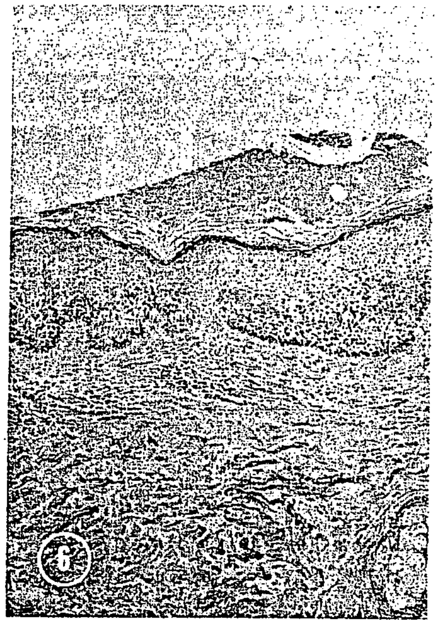
completion of treatment. Systemic side effects (i.e., flulike symptoms) were treated and controlled with oral acetaminophen.

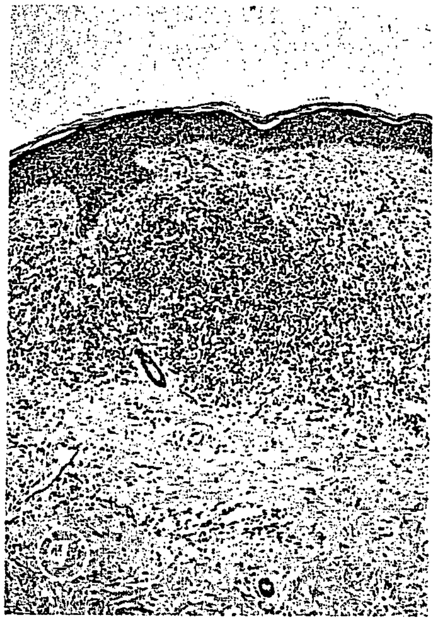
Response criteria. Excisional biopsy was performed on each of the eight lesions 8 weeks following completion of therapy (11 weeks from the start of the study). The excisional biopsy specimens were serially sectioned at 2-mm intervals, embedded in paraffin, and then serially sectioned at 4 μ. All histopathologic slides were stained with hematoxylin and eosin. Clinical responses were measured during treatment and follow-up visits through evaluation of changes in lesion size, erythema, and percentage of flattening (nodular lesions only).

CASE REPORTS

The characteristics of the cases are summarized in Table I. No residual basal cell carcinoma was present in any of the eight excisional biopsy specimens. Additionally, on Patient 1 only, an extra layer was taken and processed via the Mohs' technic and there was no evidence of tumor. Figs. 1 and 2 illustrate pre- and







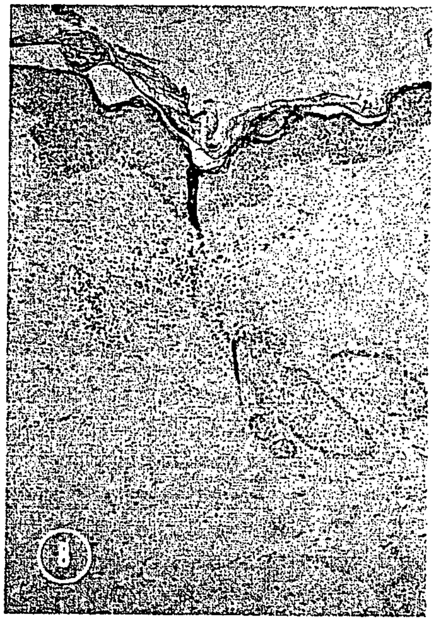


Fig. 5. Photomicrograph of pretreatment biopsy specimen from Patient 4 showing nodular basal cell carcinoma. (Hematoxylin-eosin stain; ×40.)

Fig. 6. Photomicrograph of pretreatment biopsy specimen from Patient 5 showing superficial basal cell carcinoma. (Hematoxylin-eosin stain; ×40.)

Fig. 7. Photomicrograph of posttreatment excisional biopsy specimen from Patient 4 showing focal lymphohistiocytic infiltrate. No residual basal cell carcinoma is noted. (Hematoxylin-eosin stain; ×40.)

Fig. 8. Photomicrograph of posttreatment excisional biopsy specimen from Patient 5. No residual basal cell carcinoma is noted. Note superficial location of lymphocytic and histiocytic infiltrate. (Hematoxylin-eosin stain; ×40.)

Table II.

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Table II. Adverse experiences during treatment*

Patient No.	1	2	3	4	5	6	7	8	Total
Fever (post first dose)	+	+	+	+	+			+	6
Fever (post other doses)			+	+		+			3
Malaise	+				+	+	+	+	5
Itching (at site)		+		+			+		3
Lightheadedness			+						1
Pain (at site)				+					1
Muscle aches				+				+	2
Joint aches				+			+		2
Depressive mood				+					1
Headache					+		+		2
Abdominal discomfort					+				1
Chills							+		1

*All of the adverse experiences were mild or moderate.

posttreatment appearance of the superficial basal cell carcinoma in Patient 5. Figs. 3 and 4 demonstrate the pre- and posttreatment appearance of the nodular basal cell carcinoma in Patient 6.

Histopathologic study. Pretreatment incisional biopsies confirmed the diagnosis of basal cell carcinoma in each case. The study of Cases 2, 4, and 6 (Fig. 5) revealed nodular basal cell carcinoma, while the remainder of the cases (1, 3, 5, 7, 9) were of the superficial type basal cell carcinoma (Fig. 6).

Histopathologic findings in the excisional biopsy specimens performed after the completion of the injections were reviewed by two of us (H. T. G., G. M. B.). Two initial slides with multiple serial sections were evaluated. To assure that the biopsy specimen was free of even microscopic foci of basal cell carcinoma, ten slides with additional deep serial sections were examined. No basal cell carcinoma was found in any of the initial or additional sections of the excisional biopsy specimens (Figs. 7 and 8). The sites of previous basal cell carcinoma demonstrated ectasia of vessels in the papillary dermis, perivascular lymphocytic and histiocytic accumulations, and, in some cases, lymphocyte exocytosis, Civatte body formation, and incontinence of pigment. No "tumor" stroma, necrotic basal cell carcinoma, polymorphonuclear leukocytes, transformed lymphocytes, nor eosinophils were found.

Adverse experiences. Adverse experiences during treatment occurred in all patients, with fever being the most common (Table II). Malaise was present in five patients. Other side effects included lightheadedness, muscle aches, joint aches, headaches, abdominal discomfort, chills, depressive mood, and itching and pain at the treatment site. All adverse reactions were mild or moderate, transient, and reversible, and all patients were able to complete the study. Three patients (2, 5, and 7) experienced a diminished white blood count below the normal range (4.5-11.0 K/mm^3). In each case the white blood count returned to normal during or prior to completion of the study. No other laboratory abnormalities were noted on other hematologic, chemistry, or urinalysis evaluations during or after treatment. Patient 7 experienced crushing chest pain 11 days after the last intralesional dose and underwent subsequent 5-vessel bypass for long-standing coronary artery disease. This was believed not to be related to the interferon injections. The patient recovered fully. Acetaminophen was used for side effects except for local reactions at the sites of injection, which were treated with cool compresses. (Additionally, in Patient 7 topical hydrocortisone was used for itching at the treatment site.) The side effects we observed were those to be expected with the use of intralesional interferon. Patients were observed for 4 hours after the first injection and 1 hour after subsequent injections. Side effects were more frequent with the first injection and became fewer as the study progressed. The toxicity of interferon appears to be dose-dependent with respect to at least some of its manifestations. Toxicity may also vary according to route of administration just as the effectiveness will vary. Toxicity seems to be related to the dosage, route of administration, and frequency of administration. Evening administration may reduce toxicity and side effects.[11]

DISCUSSION

The results in these eight cases clearly illustrate the marked effectiveness of injected intralesional alpha-2 interferon in the treatment of nodular and superficial basal cell carcinoma.

Lesions were noted to decrease in size during treatment and preexcisional biopsy in a range of 52% to 100% (average, 77%). Nodular lesions (3) were noted to be completely flattened in each case prior to excisional biopsy (Table I).

The mechanism of action by which interferon acts on basal cell carcinoma is unknown. Interferon has been found to have antiproliferative and immunomodulatory effects[12,13] as well as other actions.[14,15] The earliest anticancer work was performed with oncogenic viruses with subsequent inhibition of viral replication and cell transformation. In examining the cell cycle it has been found that the antiproliferative effects are not specific for a particular stage. Interferons may function as lymphokines and may depress lymphocyte proliferation. They appear to augment lymphocyte cytotoxic responses and have been used in the treatment of melanoma. While yielding limited results, the findings in these studies may be part of the reasoning behind the selection of melanoma (along with breast cancer, myeloma, and lymphoma) by the American Cancer Society to receive additional funding for further clinical trials with interferon.[16]

Response rates in malignant melanoma, while only in the 2% to 15% range, along with a 25% to 40% response rate in Kaposi's sarcoma,[17] have been of interest to dermatologists. The complete disappearance of the basal cell carcinoma in each of the eight patients may indicate a direct effect against the basal cell carcinoma, at least the nodular and superficial types. Additionally, the finding of cells from the immune system in some posttreatment biopsy specimens suggests that an immune mechanism may be operative. Interferon may therefore have properties both as a cytotoxic agent and as a modulator of the immune response.

The cosmetic results obtained were most acceptable to the patients. During the course of treatment there was an increase in the amount of erythema during treatment in all but two patients (Cases 2 and 8). During the posttreatment period, erythema decreased and at the time of excisional biopsy was mild in four patients and absent in four (compared to a pretreatment erythema evaluation of mild in seven patients and moderate in one). It is unknown when this residual erythema would have resolved. In no case did ulceration or necrosis develop during or after treatment.

The intralesional injections were well tolerated by every patient. There was a minimal amount of discomfort at the time of injection. Patients in this group were most interested in a nonsurgical approach to their lesions.

The minimum amount of alpha-2 interferon required for intralesional injection and the minimum number of doses required for its use in eliminating basal cell carcinoma are as yet unknown, but these trials are currently in progress in a multicenter study, including our institution. While we have demonstrated efficacy in nodular and superficial basal cell carcinomas, other histologic types of basal cell carcinoma, such as sclerosing basal cell carcinoma, will need to be evaluated separately. The response of lesions in specific anatomic locations such as the inner canthus of the eye and the nasolabial fold, where tumors may invade more deeply, also must be evaluated. Do anatomic areas with more of a normal sebaceous gland component (i.e., the nasal tip) hold special problems? Finally, does alpha-2 interferon indeed eliminate basal cell carcinoma completely and permanently or is there some element of suppression involved by which the lesion may redevelop at that site after a period of time?

Our results in this pilot study indicate that intralesional injection of alpha-2 interferon is a safe, effective treatment for superficial and nodular basal cell carcinoma that yields excellent cosmetic results. If results of further studies substantiate the results in this pilot study, then alpha-2 interferon therapy may be ideal for patients who are not candidates for simple surgical removal, have a nonresectable basal cell carcinoma, or desire nonsurgical therapy.

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Regression of Basal Cell Carcinoma by Intralesional Interferon-alpha Treatment Is Mediated by CD95 (Apo-1/Fas)–CD95 Ligand–induced Suicide

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Abstract

Basal cell carcinoma (BCC) is the most common skin cancer in humans, and although metastasis rarely occurs, the tumor cells are nevertheless able to invade and destroy the surrounding tissue. Intralesional injection of IFN-alpha has been found to be highly effective in inducing BCC regression by an unknown mechanism. We show that in untreated patients, BCC cells express CD95 ligand, but not the receptor, which may allow tumor expansion by averting the attack of activated CD95 receptor–positive lymphoid effector cells. The CD95 ligand of BCC cells is functional as CD95-positive cells incubated on BCC cryosections become apoptotic and are lysed. In IFN-alpha–treated patients BCC cells express not only CD95 ligand but also CD95 receptor, whereas the peritumoral infiltrate that mainly consists of CD4+ T cells predominantly contains CD95 receptor and only few CD95 ligand–positive cells. Thus, in treated patients BCC most likely regresses by committing suicide through apoptosis induction via CD95 receptor–CD95 ligand interaction. (*J. Clin. Invest.* 1997. 100:2691–2696.) Key words: basal cell carcinoma • apoptosis • CD95 • CD95 ligand • IFN-alpha

Introduction

Results from several clinical trials suggest that interferon is effective in the treatment of basal cell carcinoma (BCC)[1] (1–9). Using intralesional IFN-alpha over a 3-wk period the overall success rate in most trials was between 70 and 100% (3, 10). The mechanism by which intralesional IFN-alpha produces regression of BCC is not clear. Interferons are a group of naturally occurring glycoproteins that possess multiple biological effects including the control of cell growth and differentiation, regulation of cell surface antigen expression, and modulation of humoral and cellular immune responses (11–13). The fact that a considerable increase in the number of CD4+ T cells infiltrating the dermis and surrounding the BCC nests was observed after intralesional IFN-alpha therapy has been interpreted to indicate that this T cell subset is involved in triggering the immune response against tumor cells (2, 13, 14). Both CD4+ and CD8+ T cell subsets can express cytolytic activity against a variety of target cells, including tumor cells, by inducing apoptosis (15–17). Apoptosis is characterized by nuclear and cytoplasmic condensation of single cells with dense aggregates of chromatin lining the nuclear membrane, followed by loss of the nuclear membrane and fragmentation of the nuclear chromatin, resulting in formation of multiple membrane-bound apoptotic bodies (18–20). Recently, it has been shown that CD4+ cytotoxic T cells are capable of inducing apoptosis in their target cells via CD95 receptor–CD95 ligand interaction (21–24). CD95 receptor (CD95), a cell surface molecule belonging to the TNF receptor superfamily, is expressed on a variety of cell types, whereas CD95 ligand (CD95L), a member of the TNF family, is predominantly expressed on activated T cells (25). Thus, it is conceivable that after intralesional treatment with IFN-alpha, infiltrating cytotoxic lymphocytes destroy BCC cells by CD95–CD95L interaction. Surprisingly, we found that BCCs commit suicide upon intralesional IFN-alpha treatment by concomitant expression of CD95 as well as CD95L.

Methods

Patients. 15 patients with histologically proven nodular BCC participated in the study. Nine patients were treated with IFN-alpha, whereas six patients served as controls and remained untreated. After informed consents were obtained, lesions were injected with recombinant IFN-alpha 2b (supplied as a lyophilized powder containing 3 × 10^6 IU; INTRON-A, Essex Chemie AG, subsidiary of Schering-Plough Corp., Kenilworth, NJ). Each vial was diluted with 1 ml preservative-free sterile water. Six patients received 1.5 × 10^6 IU of IFN-alpha three times a week for 3 wk, for a total dose of 13.5 × 10^6 IU. Two patients received 3.0 × 10^6 IU per injection three times weekly with a cumulative dose of 27.0 × 10^6 IU, and another patient was treated with three injections of 1.5 × 10^6 IU given in 1 wk. Seven of the nine patients were completely cured 6 wk after completion of the therapy. In two patients the result of the therapy could not be evaluated as the tumors were excised at the end of the therapy.

Skin biopsy specimens. Skin biopsy specimens were obtained from lesional skin at the end of treatment from two patients. In one patient biopsy was performed 1 wk after completion of the therapy, and six patients had skin biopsies 14 to 21 d after treatment. Excisional biopsies from six untreated patients with nodular BCCs served as controls. All tissue specimens were snap-frozen in liquid nitrogen and used for immunohistochemical staining and for in situ apoptosis detection.

Immunohistochemistry. The monoclonal antibodies used were CD3, CD4, CD8 (Dako SA, Glostrup, Denmark), and anti-CD95 (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). A polyclonal anti-CD95L-peptide antibody has been prepared in rabbits. The peptide selected according to Tanaka et al. (26) was synthesized and coupled via cysteine to maleide-activated keyhole limpet hemocyanin or ovalbumin (KLH-CD95L; OVA-CD95L) using the Imject Activated Immunogen Conjugation Kit (Pierce, Rockford IL). A rabbit was intramuscularly injected with 500 μg KLH-CD95L emulsified in complete (for the first injection) or incomplete Freund's

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[1] *Abbreviation used in this paper:* BCC, basal cell carcinoma.

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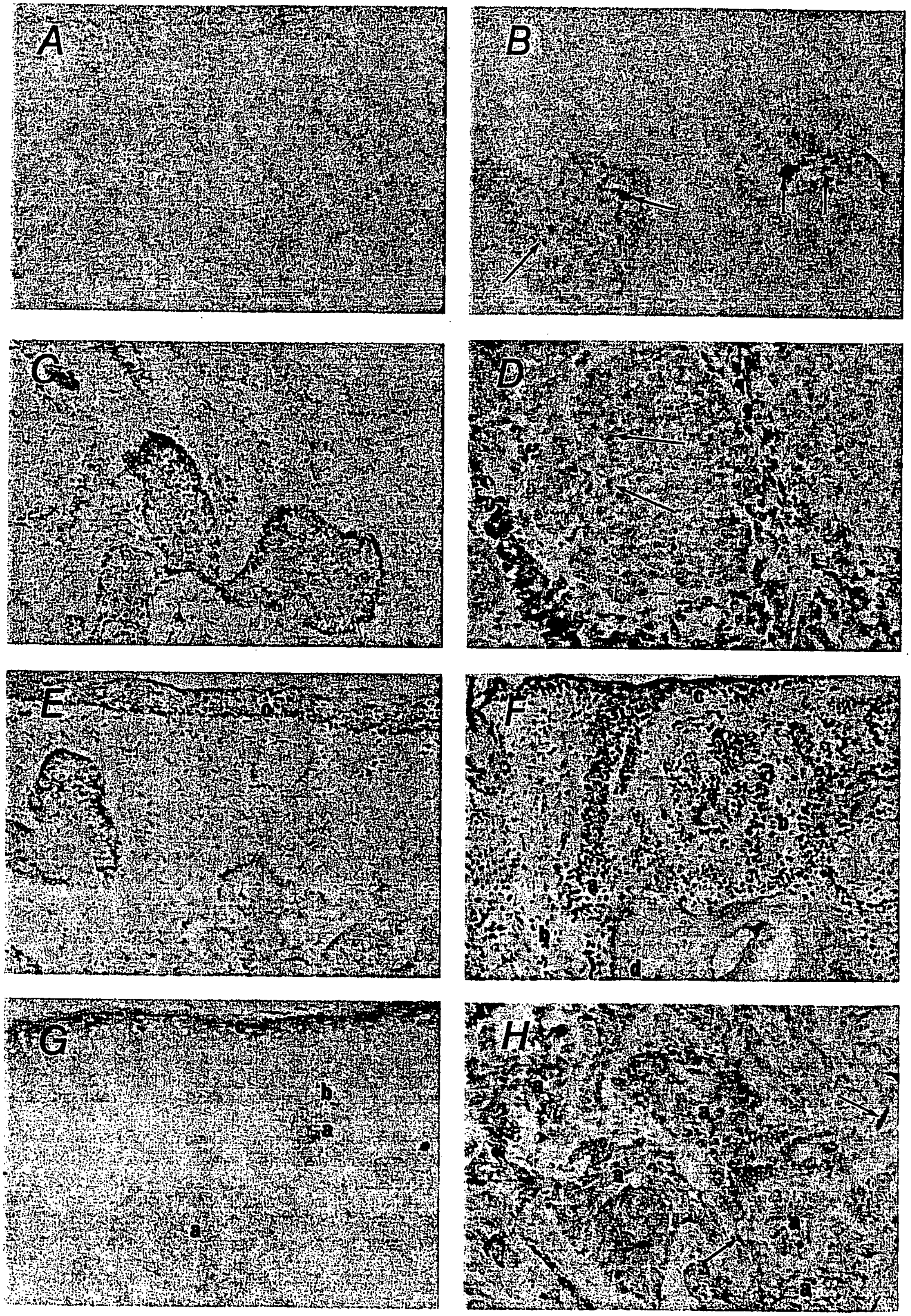

Figure 1. Apoptosis, expression of CD4, CD95, and/or CD95-ligand in BCC of IFN-alpha–treated or –untreated patients. (*A*, *C*, *E*, and *G*) Untreated patients; (*B*, *D*, *F*, and *H*) IFN-alpha–treated patients. (*A*) No apoptosis is found in BCC of untreated patients. (*B*) Apoptotic cells (*dark brown*) are found in the center of the tumor nests (*arrows*) of IFN-alpha–treated BCC. (*C*) A sparse lymphoid infiltrate consisting of CD4+

adjuvant four times at three weekly intervals. Blood collection was done 7 d after the last injection. The serum obtained was affinity-purified over OVA-CD95L coupled to CNBr sepharose (Pharmacia Biotech, Dübendorf, Switzerland) or over CD95L peptide bound to Ultralink iodoacetyl matrix (Pierce, Rockford, IL). The specificity of the purified antibody was assayed in enzyme immunoassays using soluble human CD95L (Alexis Corporation, Läufelfingen)-coated plates on CD95L-positive cell lines (Jurkat, HUT) by immunoperoxidase staining, Western blots, and cytofluorography.

The immunohistochemical staining procedure used involving the monoclonal antibodies was the labeled streptavidin–biotin technique (Dako Diagnostics AG, Zug, Switzerland). Frozen specimens were cut in 6 µm–thick cryostat sections, air-dried, and fixed in acetone for 10 min at −20°C. Sections were then incubated for 30 min at room temperature with a primary murine monoclonal antibody at a concentration of 1:100. Endogenous peroxidase activity was blocked by incubation with 0.6% hydrogen peroxide in methanol. The sections were subsequently incubated with biotinylated goat anti–mouse Ig as secondary antibody at a concentration of 1:100 for 25 min. After washing in Tris-HCl buffer, the sections were incubated with peroxidase-conjugated streptavidin (1:100) as a third reagent for 25 min. Peroxidase activity was visualized using 3-amino-9-ethyl carbazole solution (AEC substrate-chromogen; Dako Diagnostics AG) according to the manufacturer's instruction. Finally, sections were gently washed with distilled water, counterstained with haematoxylin, and mounted under glass coverslips. In each case and for each antibody, omission of the primary antibody was used as a negative control. For staining with the anti-CD95L antibody, acetone-fixed sections were preincubated in PBS containing 10% goat serum at 37°C for 30 min. Sections were then incubated with the affinity-purified anti-CD95L at a concentration of 1:15 at room temperature for 1 h, washed in PBS, and incubated either with a 1:200 dilution of peroxidase-conjugated goat anti–rabbit IgG (Sigma Chemie AG, Buchs, Switzerland) at room temperature for 30 min, or with biotinylated goat anti–mouse Ig followed by peroxidase-conjugated streptavidin as described above. As control, the prebled serum of the same rabbit used for the production of anti-CD95L was used at the same concentration.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The Apop Tag (Oncor Inc., Gaithersburg, MD) in situ apoptosis detection kit or the In Situ Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instructions. In brief, tissue cryosections were fixed in formalin for 10 min, washed, and post-fixed in a 2:1 mixture of ethanol and acetic acid for 5 min at −20°C. Endogenous peroxidase was blocked in 2% hydrogen peroxide. After rinsing and applying equilibrium buffer, TdT enzyme was added and incubated at 37°C for 1 h. Stop/wash buffer was then added for 10 min, and antidigoxigenin peroxidase was applied for 30 min. Color development was done using DAB substrate solution, and counterstaining was performed using methyl green. As negative controls, the slides were prepared in the same way except that TdT enzyme was omitted. As positive control, an IL-3 dependent mastocytoma cell line was used, which becomes rapidly apoptotic upon IL-3 removal. The Boehringer kit was used in a similar protocol except that after blocking endogenous peroxidase the slides were permeabilized with 0.1% Triton, and fluorescein-dUTP was used to label DNA strand breaks.

Demonstration of functional CD95L. Cryosections were prepared from IFN-treated and untreated BCC as well as from healthy skin, and were transferred onto siliconized glass slides. CD95-positive cells (4 × 10^5 A20.2J) were seeded on each cryosection in a half-and-half mixture of Iscove's modified Dulbecco's medium containing 5% FCS and keratinocyte-SFM medium (Life Technologies AG, Basel, Switzerland). After 6 h the cells were harvested, washed with PBS, fixed in 70% ethanol for 1 h at −20°C, and stained with propidium iodide (50 µg/ml) for 15 min at 37°C. Quantitative analysis of apoptosis was performed by FACScan (Becton Dickinson, Basel, Switzerland) according to a published method (27). Data analysis was performed with Cell Quest software (Becton Dickinson). As further controls, A20.2J were incubated onto the same glass slides with soluble recombinant murine CD95L (kindly obtained from Dr. A. Fontana, University Hospital Zürich) or with medium alone. The experiments were performed three times with several IFN-treated and nontreated patients. To assess CD95L-mediated lysis, ^{51}Cr-labeled A20.2J (3 × 10^5) were seeded onto the cryosections as described above. After 24 h supernatants (50 µl) were harvested and tested for ^{51}Cr release. Details of the ^{51}Cr release assay and calculation of lysis are given elsewhere (21). Two independent experiments were done using cryosections of treated and nontreated patients.

Results

Identification of apoptosis by in situ end-labeling of fragmented DNA. Biopsy specimens taken from six untreated patients showed that virtually no apoptotic cells were found in BCCs and the small lymphoid infiltrate as identified by the TUNEL (28) technique (Fig. 1 *A*). In contrast, numerous single apoptotic cells were identified within the tumor masses in patients treated with intralesional IFN-alpha (Fig. 1 *B*). Interestingly, apoptosis was especially manifest in the center and less at periphery of the tumor nodules (Fig. 1 *B*, *arrows*) and the number of apoptotic cells was particularly high in cases showing advanced regression of tumor nests. A small but significant number of apoptotic cells was also manifest in the keratinocytes of the epidermis in the treated as well as untreated patients (data not shown).

Investigation of BCC, the surrounding tissue, and the lymphoid infiltrate for CD95 and CD95L by immunohistochemical staining. Biopsy specimens taken from nine interferon-treated BCCs revealed a dense dermal lymphoid infiltrate surrounding the regressing tumor nests. The number of cells in the peritumoral infiltrate was highest in biopsy specimens taken from the lesions 2–3 wk after the completion of therapy. Immunohistochemically, the majority of cells in the peritumoral infiltrate were CD4+ T cells (Fig. 1 *D*). Only few T cells were found within the tumor nodules (*arrows*). Approximately 10–20% of the peritumoral infiltrate were CD8+ T cells (data not shown). In contrast, lymphoid-infiltrating cells were only sparsely present in the dermis of patients with untreated BCC

Figure 1 legend (Continued)

T cells is found in the dermis of untreated patients. (*D*) CD4+ T cells surround the BCC nests in treated patients, but only few T cells are found within the tumor nodules (*arrows*). (*E*) In untreated patients only the keratinocytes in the basal cell layer express CD95 (*c*), whereas the BCC are CD95-negative. (*F*) In treated patients a large number of CD95-positive cells are found within the tumor nests (*a*), in the lymphoid infiltrates surrounding the tumors (*b*), and in the basal cell layer of the epidermis (*c*). Epidermic cysts (*d*) presumably derive from the apoptosis and destruction of the tumor masses. (*G* and *H*) BCC express CD95L in IFN-alpha–treated (*H*) as well as untreated patients (*G*). CD95L-positive cells are predominantly located in the center of the tumor nests (*a*), some at the periphery of the tumor nests (*b*), and some in the spinous layers of the epidermis (*c*). Few CD95L-positive cells are found in the infiltrate preferentially near the blood vessels (*arrows*). All figures are at an original magnification of 32 with the exception of *D* and *H*, which are at a magnification of 80.

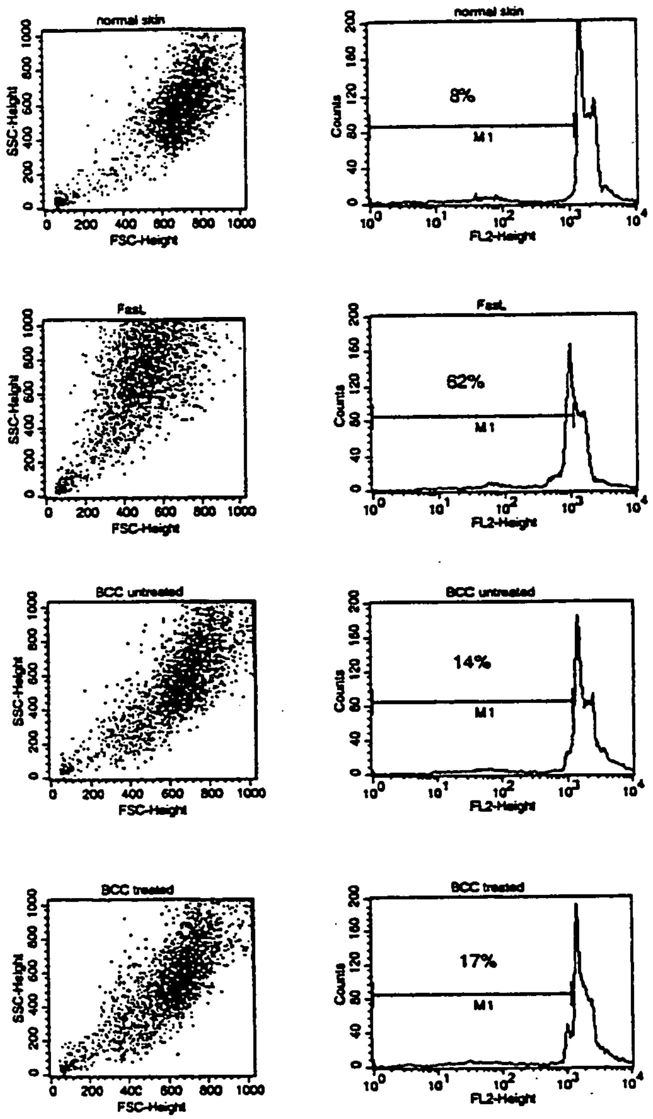

Figure 2. BCC induces apoptosis in CD95-positive A20.2J cells. A20.2J were added onto cryosections of normal skin or BCC from IFN-alpha–treated and untreated patients for 6 h. The cells were then harvested, fixed, and stained with propidium iodide. The rate of apoptosis was analyzed by FACScan. As a control, A20.2J were incubated with soluble CD95L (*FasL*). The negative control, A20.2J alone, is not shown as no apoptosis was detected. The propidium iodide staining and the percentage of apoptotic cells is given in the histograms. *Dot blots* note the marked cell shrinking (as a sign of apoptosis) of the A20.2J incubated on treated or untreated BCC compared with normal skin.

(Fig. 1 *C*), mostly around the blood vessels and at the periphery of tumor nodules. A similar sparse distribution of CD95-positive cells was found in the dermis of six untreated patients, whereas the BCC cells were completely CD95-negative (Fig. 1 *E*). Marked focal inter- and intracellular staining with anti-CD95 (Fig. 1 *F*), however, was seen on the tumor cells in all BCCs treated with intralesional IFN-alpha. In addition, CD95 was strongly expressed on infiltrating lymphoid cells at the periphery of the tumor nests and on those in close contact with the tumor cells (Fig. 1 *F*). Strong expression of CD95L-positive cells was detected within and around the BCC nodules in all untreated patients (Fig. 1 *G*). In IFN-alpha–treated patients the BCC tumor nests demonstrated intense CD95L-expression (Fig. 1 *H*), whereas only few CD95L-expressing cells were found in the lymphoid infiltrates preferentially in the area of blood vessels (*arrows*). Thus, while the BCC cells of untreated patients constitutively expressed CD95L, the BCC cells of treated patients expressed both CD95L as well as CD95 receptor.

Of interest, basal and suprabasal keratinocytes within the spinous layer strongly stained with anti-CD95 antibody not only in patients with BCC (Fig. 1, *E* and *F*), but also in healthy control subjects (data not shown). In contrast to CD95, CD95L was strongly expressed on keratinocytes in the spinous layer (Fig. 1 *G*).

BCC express functional CD95L. To evaluate whether the CD95L expressed by BCC from IFN-alpha–treated and –untreated patients is functional, CD95-positive cells (A20.2J) were incubated on BCC-cryosections, and apoptosis and lysis were measured. FACS analysis after propidium iodide staining showed an increase in the amount of apoptosis of A20.2J added for 6 h onto BCC from untreated as well as IFN-alpha–treated patients (Fig. 2). Normal skin also induced a low amount of apoptosis, which is not surprising since keratinocytes of the spinous layer are CD95L-positive. Moreover, the A20.2J cells incubated on BCC cryosections for 24 h were strongly lysed as demonstrated in the more sensitive ^{51}Cr release assay (Table I), independent of whether the BCC originated from IFN-alpha–treated or –untreated patients. Normal skin also induced some lysis of A20.2J, again reflecting upon CD95L expression of the keratinocytes.

Discussion

The results of our study demonstrate that apoptosis is the major mechanism of tumor cell death in regressing BCC after intralesional IFN-alpha treatment. Although numerous factors such as chemotherapeutic agents, ionizing radiation, oncogenes, glucocorticoids, tumor necrosis factor, transforming growth factor beta, and cytolytic T cells are known to induce

Table I. BCC from Treated and Untreated Patients Lyse CD95⁺ Cells

	^{51}Cr release of A20.2J	
	Exp I	Exp II
	cpm	*cpm*
Total release	12204	11915
Spontaneous release	3173	3069
Soluble CD95L	n.t.	7819 (54%)
Normal skin	4839 (18%)	5765 (30%)
BCC Pat. A untreated	7941 (53%)	9612 (74%)
BCC Pat. B treated	6998 (42%)	8261 (59%)
BCC Pat. C treated	7826 (51%)	n.t.

^{51}Cr-labeled A20.2J were added to cryosections of normal skin or BCC obtained from IFN-alpha–treated or –untreated patients. After 24 h supernatants were harvested and the ^{51}Cr release was determined as cpm (% specific release in parenthesis). As control, soluble recombinant CD95L was added to ^{51}Cr-labeled A20.2J. n.t., not tested; Pat, patient.

apoptosis (for review see reference 29), the CD95-CD95L system has been identified as the most important trigger of apoptosis. In this study, we found that the BCCs of untreated patients were strongly CD95L-positive. CD95 was not detectable, confirming the results of other groups (30, 31). Thus, BCC formation in untreated patients may be due to the ability of the tumor cells to lyse attacking CD95-expressing effector T cells via their CD95L. Indeed, CD95L of the BCC proved to be functional and induced in vitro apoptosis and lysis of CD95-positive cells such as A20.2J. This strategy resembles the proposed role for CD95L in the maintainance of immune privilege in mouse testis and in the anterior chamber of the eye (32, 33). Furthermore, our observation is in close agreement with the recent demonstration of constitutive CD95L expression on large granular lymphocytic leukemia cells (34), colon cancer cells (35), melanoma cells (36), hepatocellular carcinoma cells (37), and astrocytoma cells (38). No CD95 expression was detected on colon cancer and melanoma cells, whereas the hepatocellular carcinomas apparently lost CD95 in contrast to normal liver cells (35–37). These tumor types have been suspected to expand by the induction of apoptosis in infiltrating CD95-positive lymphocytes. In our study, however, as the consequence of intralesional treatment with IFN-alpha, BCC cells not only expressed CD95L but also became CD95-positive. Hence, the concomitant expression of both CD95 and CD95L could induce apoptosis within the tumor cells, eventually leading to cell death by suicide or fratricide. A similar mechanism has been reported in T cell leukemia lines by Friesen et al. (39), who found in in vitro studies that some anticancer drugs (doxorubicin, methotrexate) induced apoptosis via upregulation of the CD95-CD95L system. The question arises whether the infiltrating lymphoid cells also contribute to the destruction of BCC. Indeed, upon IFN-alpha treatment a large lymphoid infiltrate mainly consisting of CD4+ T cells becomes manifest, predominantly around the tumor nests. The majority of these cells infiltrating the dermis, however, express CD95, and only few express CD95L. Although CD4+ CTLs may be able to induce apoptosis in BCC cells via engagement with CD95 (21–23, 25, 40), a major lytic effect of such CD95L-positive CTLs on BCC is rather unlikely for the following reasons: first, only very few T cells infiltrate the tumor nests, the large majority being located around the BCC (see Fig. 1 *D*); and second, apoptosis is manifest in the center (see Fig. 1 *B*) and not at the periphery of the BCC nests, suggesting that tumor destruction starts from inside the nodules, eventually leading to epidermic cysts.

What role does IFN-alpha play in BCC regression? Although IFN-alpha is claimed to inhibit the growth and proliferation of malignant cells by prolonging the length of the cell cycle (11), we propose that its major antitumor property may be based on the recruitment of infiltrating cells and/or upregulation of the CD95-CD95L system. Such an effect may also be responsible for the induction of apoptotic cell death in squamous cell carcinoma cell lines by IFN-alpha (41). Presently, it is unknown whether the IFN-alpha–induced CD95 expression on BCCs is a direct effect of the drug, or is indirectly mediated, e.g., by the lymphoid effector cells. It has been shown that IFN-alpha increases the frequency of IFN-gamma–producing CD4+ T cells (42), and significantly expands the number of CD4+ T cells in the dermis (43). It is also well established that CD95 expression can be induced by interleukin-2 or IFN-gamma, or by a combination of IFN-gamma and TNF-alpha (25). Recent studies showed that IFN-gamma induces a CD95-dependent apoptotic process in keratinocytes (44, 45). Since CD4+ T cells that are known to be a potent source of such cytokines represent the bulk of the infiltrating peritumoral lymphocytes, it is possible that they upregulate CD95 on BCC cells by secreting these cytokines, and thus indirectly contribute to the CD95L-based cytotoxicity. Presently ongoing in vitro studies will help to better define these unresolved issues.

In summary, our findings reveal the mechanism of BCC regression upon intralesional IFN-alpha treatment. The apoptotic cell death in BCC, as identified by DNA fragmentation and followed by tumor regression, results from specific CD95-CD95L interactions. Our study demonstrates that certain tumors can be treated successfully by modulating the CD95-CD95L-system.

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Interferon-α as an immunotherapeutic protein

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Abstract: Interferon-α (IFN-α) has proven to be a clinically effective antiviral and antineoplastic therapeutic drug for more than 16 years. During this time, evidence from in vitro laboratory studies and the clinical arena has supported the concept that IFN-α is an immunotherapeutic drug. By regulating a diverse set of cytokines and their receptors, IFN-α is uniquely positioned to prime the host immune response and provide an effective antineoplastic- and antiviral-immune response. IFN-α stimulates the innate cell-mediated response and then participates in the transition of the initial host innate response into an effective adaptive-immune response. IFN-α also drives the adaptive cell-mediated CD8+ T-cell response and helps to maintain a CD4+ Th1-cell population balance for an effective antineoplastic and antiviral host defense. This review will describe the current state of knowledge of IFN-α as an immunoregulatory protein and address specific issues of IFN-α as an immunotherapeutic for antineoplastic and antiviral diseases. *J. Leukoc. Biol.* 71: 565–581; 2002.

Key Words: antiviral therapy · antitumor therapy · immunological therapy

INTERFERON-α (IFN-α), THE QUIET IMMUNE-REGULATORY CYTOKINE

First described as a protein secreted by fibroblasts, the IFN-α cytokine was shown to induce paracrine resistance against lytic virus infection [1]. Yet, an active virus infection was not required solely for the induction of IFN-α, because expression could also be induced by the treatment of cells with endotoxins, dsRNA, poly (I:C), or CpG [2–5]. Later, an IFN-α homologue was identified from lymphoid cells, the IFN-γ protein that could also be expressed by nonlymphoid cells [6, 7]. These experiments led to the classification of IFN-α and IFN-β as Type I and IFN-γ as Type II IFNs. It now appears that IFN-α/β and IFN-γ have nonredundant and functionally complementary activities in the host response to viral infection [8–10].

A thorough analysis of IFN-α expression shows that IFN-α is secreted not only by fibroblasts but also by T cells, macrophages, plasmacytoid monocytes, dendritic cells (DCs), and natural killer (NK) cells [2–4, 11, 12]. More recently, IFN-α has been classified as the "leukocyte interferon" [13]. This designation was intended to refer to the principal host cell secreting the IFN rather than to serve as a comment on the cytokine's immunologic mechanism of action. The current working model for IFN-α proposes that the "professional" IFN-α/β secretor cells are the CD4+CD11c-type 2 DC precursors (pDC2s) [14–16]. The pDC2 cells secrete between 200 and 1000 times more IFN-α than any other white blood cell [16]. Because DCs are important to the migration of activated T cells to injured/infected tissue, IFN-α is involved indirectly in regulation of the local immune response [17]. The putative role of the pDC2 also highlights an important aspect of IFN biology: that the regulation of patient-innate and adaptive-immune responses to IFN-α therapy may vary depending on dose and administration schedule. This concept serves to emphasize the potentially important role that a low-dose, systemic, therapeutic administration of IFN-α during antineoplastic or antiviral treatment may serve mechanistically as a priming cytokine for the host immune response [15, 18].

IFN-α primes the host immune response

IFN-α and IFN-γ are important to host immune defense against neoplastic and viral diseases [8–10]. Biron [19] was perhaps the first reviewer to state clearly, "IFN-α/β play a dominant role in shaping downstream innate and adaptive immune responses to viral infection" (**Fig. 1**). Because IFN-α expression occurs as an early response to infection, it precedes a majority of the other innate-immune response cytokines. In fact, the timing of IFN-α expression after infection suggests that its primary role is to induce a priming state during the initial immune response to infection [20–22]. This IFN-α-induced priming activity is thought to augment the host primary-immune response to viral infection [18]. However, IFN-α activity on the immune system also shows an overlapping function (and in some instances, a synergy) with other "early response" cytokines [e.g., transforming growth factor-α (TGF-α) and interleukin (IL)-2; 23]. Thus, it has been suggested that IFN-α is the first and most important cytokine secreted by antigen-presenting cells (APC) after antigen stimulation by a T-helper cell type (Th)0 cell [21]. The phenomena known as priming was first shown when it was observed that a low-dose IFN-α treatment preceding viral, endotoxin, or poly(I:C) challenge resulted in an increased protection from viral challenge [3, 18]. An IFN-α-induced priming response is also seen with IL-2 production in mitogen-activation models that are enhanced by IFN-α pretreatment [23].

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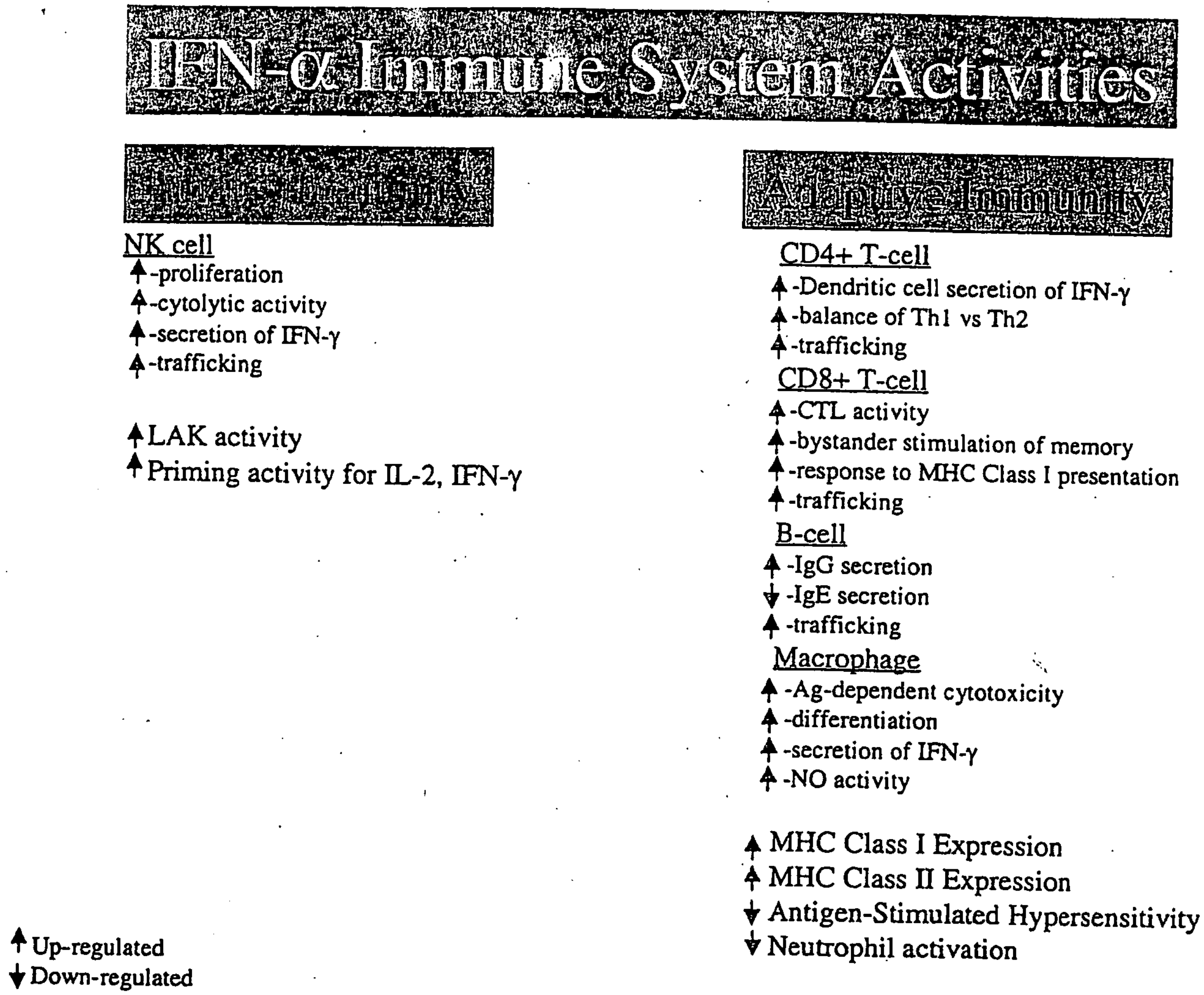
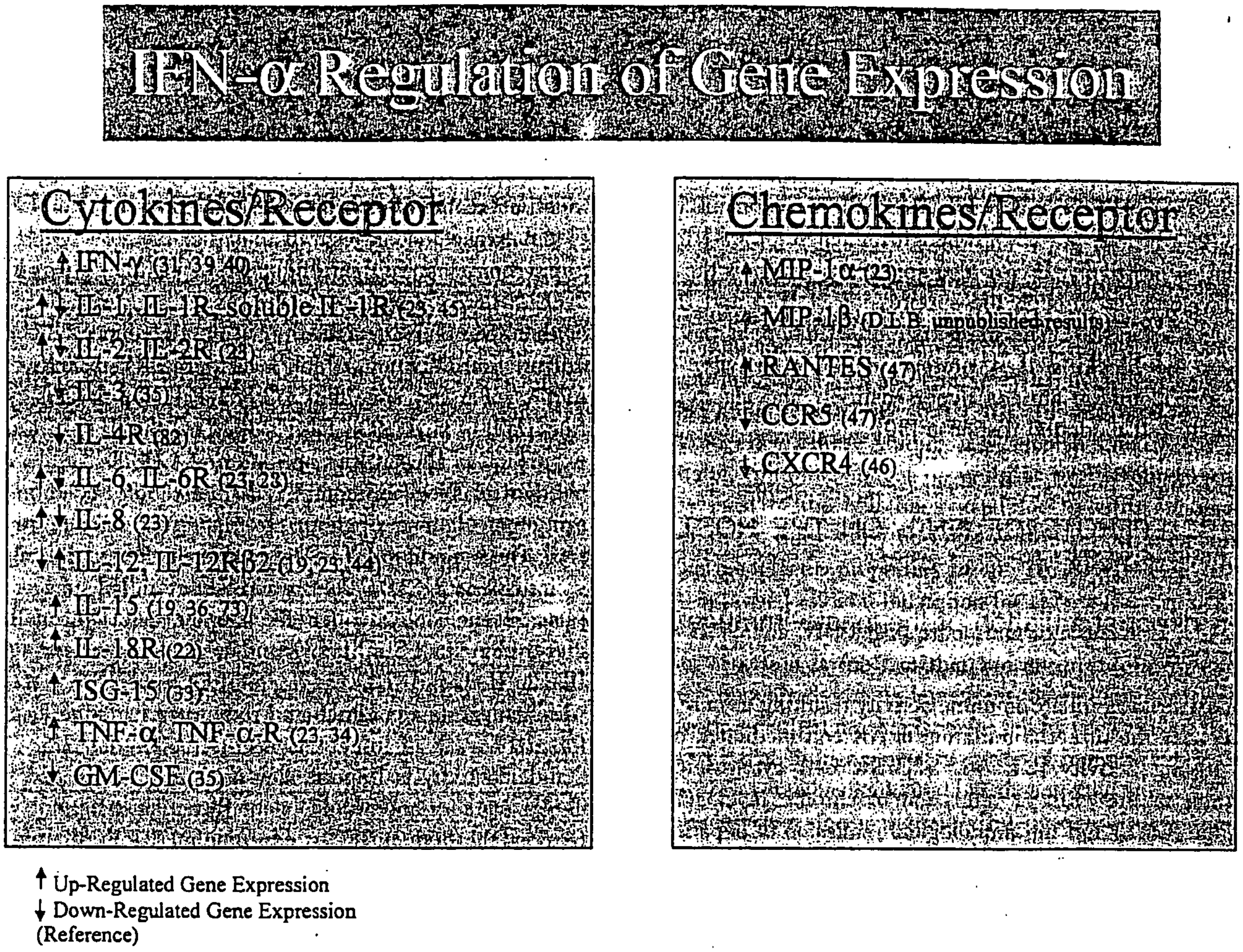


Fig. 1. IFN-α is important to the transition of the immune system from an innate response to an adaptive response. This transition is critical for the effective clearance of all nonself or foreign antigens and the maintenance of immunological memory. As shown, IFN-α affects numerous cell types including the induction of macrophage activity, NK cell cytotoxicity, and CTL activity and the decrease of neutrophil activation. These activities show overlapping function with other early response cytokines such as TGF-α and IL-2 and in some situations, can induce a synergic response.

The ability of IFN-α to prime the host immune system is consistent with the dose-dependent responses typically seen with cytokines eliciting low-dose stimulating effects and high-dose, tolerizing, or suppressant anergies [3, 23–25]. Low-dose treatment with IFN-α has been shown to down-regulate delayed-type hypersensitivity and cellular infiltration into the peripheral lymph nodes [26]. High doses of IFN-α are clinically effective against viral or neoplastic disease but are poorly tolerated by the patient [24]. In theory, low-dose treatments mimic early endogenous IFN-α priming events [27]. Studies suggest that low-dose treatment with IFN-α resembles a mucosal immune response in comparison with a systemic immune response [27]. Mucosal immunity is often referred to as local immunity, and so the distinction with IFN-α low-dose treatment is not lost. For example, the local delivery of IFN-α is responsible for NK and T-cell responsiveness to IL-12-induced secretion of IFN-γ and subsequently drives a Th1 response [19]. Additional local effects of IFN-α treatment include a block in the IL-8 attraction and activation of neutrophils at inflammation sites [23].

IFN-α links the transition of innate to adaptive immunity

IFN-α may be very important in linking the innate-immune response with the sustained adaptive-immune response [20–23, 28–30]. The innate-immune response usually consists of the cell-mediated response of NK cells to nonself (e.g., neoplastic) or foreign (e.g., viral) antigen. Although important for the initial defense of the host, the innate-immune response must transition to the more efficient and specific adaptive-immune response to clear the nonself or foreign antigen effectively. Critical to IFN-α regulation of the transition from the innate- to the adaptive-immune response is the fact that IFN-α treatment has been shown directly or indirectly to regulate the activity of other cytokines and chemokines (**Fig. 2**) [19, 23, 25]. To date, these include IFN-γ, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, IL-13, IL-15, tumor necrosis factor α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-inducible protein 10 (IP-10), and IFN-stimulated gene 15 (ISG-15) [19, 23, 31–36]. The complexity of the regulation is



IFN-α Regulation of Gene Expression

Cytokines/Receptor

↑ IFN-γ (31,39,40)
↑↓ IL-1, IL-1R, soluble IL-1R (23,45)
↑↓ IL-2, IL-2R (23)
↓ IL-3 (35)
↓ IL-4R (82)
↑↓ IL-6, IL-6R (23,23)
↑↓ IL-8 (23)
↓↑ IL-12, IL-12Rβ2 (19,23,44)
↑ IL-15 (19,36,73)
↑ IL-18R (22)
↑ ISG-15 (39)
↑ TNF-α, TNF-α-R (23,34)
↓ GM-CSF (35)

Chemokines/Receptor

↑ MIP-1α (23)
↑ MIP-1β (D.L.B. unpublished results)
↑ RANTES (47)
↓ CCR5 (47)
↓ CXCR4 (46)

↑ Up-Regulated Gene Expression
↓ Down-Regulated Gene Expression
(Reference)

Fig. 2. As an immunomodulatory cytokine, IFN-α regulates the transcription of multiple cytokine and chemokine pathways directly or indirectly. Direct transcriptional regulation occurs after ligand binding to the type I IFN receptor and the initiation of a signal-transduction cascade, which includes members of the JAK and STAT families. Because the direct transcriptional regulation is dose-, time-, and cell-type-dependent, this emphasizes the diversity of attributable IFN-α activities and the difference between high- and low-dose therapies.

demonstrated further by a synergy between IFN-α and several other cytokines, most important including IFN-β and IFN-γ [20, 23, 37, 38]. For example, IFN-α synergizes with IL-18 to induce IFN-γ gene transcription and protein expression [22]. In fact, a possible nexus for the innate-to-adaptive transition may be through the IFN-α-induced up-regulation of IFN-γ protein in CD4+ and CD8+ T cells and NK cells [31, 39, 40]. Furthermore, it has been suggested that IFN-α signal transduction is augmented and expanded by the signal-transduction capacity of IFN-γ [20, 37–39, 41, 42]. Despite these important signal-transduction responses, antagonism of IFN-α- and IFN-γ-induced transcriptional activation has been observed with cytokines such as IL-4 [43]. Together, these observations underscore the complexity of the immunological regulatory mechanisms for IFN-α.

Additional literature suggests that IFN-α regulation of the host immune system is not mediated solely through the regulation of other cytokines and chemokines, but through the regulation of cytokine- and chemokine-receptor expression at the cell surface [44]. For example, IFN-α up-regulates the IL-12Rβ2 subunit in CD4+ T cells and thus, increases APC responsiveness to IL-12 [23, 44]. To date, IFN-α has been shown to regulate the cell-surface expression or soluble expression of CXCR4, CCR5, TNF-α, IL-2R, IL-1R, IL-4R, IL-6R, and IL-18R components [22, 23, 28, 45–47, 82].

IFN-α regulates the immune response through the Th1/Th2 balance

The predisposition and regulation of the CD4+ Th population are important in determining how well the host responds to an infection or a neoplasm and if it is capable of sustaining an effective, antiseptic response over the length of the challenge [48]. Recently, the importance of the balance between the CD4+ Th1 and Th2 cell populations on immune-system function has been reviewed in-depth and will not be addressed thoroughly in this article [49, 50]. However, a putative role for IFN-α in the regulation of the Th1 response has been suggested. Specifically, in vivo IFN-α treatment promotes Th1 cell differentiation indirectly and re-establishes a Th1/Th2 population balance in diseases and infections that promote a Th2 cell imbalance [29, 51]. One possible mechanism is mediated through the IFN-α-induced up-regulation of IL-12R. During differentiation of human naïve T cells into the Th1 and Th2 subsets after stimulation with antigen, there is a selective expression of the IL-12Rβ2 subunit on Th1 cells only. In fact, IL-12 and IFN-α can induce the expression of IL-12Rβ2 in Th1 cells, thus selectively promoting a Th1 response [23, 27, 44, 52]. Furthermore, IFN-γ is a key regulatory cytokine involved in the promotion and maintenance of the Th1 cell population and is secreted by CD4+ T cells induced with IFN-α. Thus, IFN-α stimulates Th1 cell development indirectly [29–31, 51, 53–55]. Although influencing the Th1 cell population positively, IFN-α also appears to suppress the Th2 cell development through the suppression of IL-4 and IL-13 gene expression [30]. The immediate effect dampens the Th2 response by blocking IL-4 protein inducible activity, thus decreasing potential antigen hypersensitivity and maximizing the innate cell-mediated response [30].

DIRECT EFFECTS OF IFN-α ON THE HOST IMMUNE SYSTEM

IFN-α direct stimulation of the innate-immune response

The most striking innate-immune activity resulting from IFN-α treatment is the direct stimulation of NK cell-mediated, cytotoxic killing activity [34, 56, 57]. NK cell cytotoxicity is important to the clinical remission of chronic myelogenous leukemia (CML) and the pathological remission of hepatitis C virus (HCV)-infected patients [58, 59]. NK cells are one of the first professional killing cells to arrive in the early antineoplastic and antiviral immune response. Because NK cell cytotoxicity is nondirected, the activity is categorized as being a part of the innate-immune response. Locally produced IFN-α stimulates increased cytotoxic killing activity in regional NK cells, and although the mechanism of action is unclear, one aspect is mediated through a direct induction of perforin mRNA expression in CD8+ and NK cells [34, 60–62] and IFN-α has two other important regulatory effects on NK cells. IFN-α stimulates the proliferation of NK cells [25, 63]. IFN-α also enhances the production or secretion of other cytokines by the NK cell through the autocrine IFN-γ loop [34, 56, 62, 64, 65]. However, it should be noted that although IFN-α stimulates NK cell cytotoxicity, it also protects normal/uninfected cells from antibody-independent cell death by decreasing their susceptibility to nonspecific cell death [60, 66, 67]. The regulatory role of IFN-α on NK and target cells underscores both concepts for IFN-α as a requisite-priming cytokine and as a local effector molecule.

IFN-α regulation of the adaptive-immune response

Following the innate response, the host must adapt the immune system efficiently against the foreign antigen to create a strong, antigen-specific, prolonged, and regulated immune response. Adaptive immunity is initiated and driven by the proper cognate presentation to T cells of nonself, foreign antigen by the class I or class II major histocompatibility complex (MHC) on APC. Specifically, antigen presentation by the class I MHC complex or by the class II complex on a B cell stimulates the cell-mediated CD8 cytotoxic T-cell response [68].

IFN-α has been shown to effect the CD8+ T-cell and B-cell adaptive-immune response [69]. Such effects extend from a profound regulatory role by IFN-α in stimulating the proliferation, activation, and generation of existing memory CD8+ cytotoxic T cells (CTLs) to the stimulation of lymphocyte-activated killer (LAK) activity [20, 42, 70, 71]. IFN-α treatment, or treatment with inducers of IFN-α, is important for the clonal expansion and survival of the memory CD8+ T-cell population in an antigen-independent manner [70, 71]. However, this type of bystander effect is not as powerful a response as that seen characteristically with antigen-specific expansion during viral infection, which logarithmically expands the population of a few specific CD8+ T-cell populations typically from 1000- to 10,000-fold [72]. Yet, the IFN-α-stimulated expansion of the entire population of CD8+ T cells is independent of T-cell receptor (TCR) activation and represents a unique mechanism of control over adaptive-immune responses [70]. The importance of IFN-α in this effect has been suggested using IFN-α/βR knockout mice. Sun et al. [5] suggest a complete absence of bystander effects upon CpG DNA challenge in vivo and in vitro. Similar bystander effects have been seen with IL-12, IL-15, IL-18, CpG, poly(I:C), and IFN-γ treatment, which suggests a common and redundant cytokine mechanism for eradication of infectious agents [5, 36, 70, 73]. This bystander activity is important to the preservation of long-term T-cell memory and would occur during intermittent viral infections that induce IFN-α expression [72]. Furthermore, type I IFNs are also known to be involved with the nonspecific maintenance of T cells through an inhibition of apoptosis and forced quiescence in the absence of antigen [74]. Thus, IFN-α assists in antigen specificity, selection, and proliferation of CD8+ T cells during the adaptive-immune response and highlights an important interaction between the nonspecific innate-immune system and the adaptive-immune system.

Another significant activity of IFN-α identified very early from in vitro studies was the up-regulation of class I and class II MHC expression [75–77]. IFN-α up-regulates the transcription of class I MHC proteins directly, resulting in increased antigen presentation, immune surveillance, and cognate cell-mediated killing to eliminate virally infected and neoplastic cells [77, 78]. The up-regulation of class I MHC by IFN-α also promotes the development of CD8+ T-cell responses [70]. IFN-α and IFN-γ up-regulate class II MHC expression, which promotes enhanced CD4+ T-cell responses and antigen presentation [76, 79].

The role of IFN-α on the B-cell-mediated, adaptive response is more subtle. The clearest effect observed has been with immunoglobulin (Ig) production. IFN-γ and IFN-α have been shown to enhance IgG production and down-regulate IgE secretion in B cells [69, 78, 80, 81]. Under the direction of IFN-α, the Ig isotype-selection process induces a predominantly IgG2a antibody-isotype response [69]. It is suggested that IFN-α down-regulates IgE secretion through the post-transcriptional down-regulation of IL-4 and the IL-4R mRNA [82]. This IFN-α activity antagonizes IL-4-driven, Th2 immune responses and may also compliment the IFN-α stimulation of macrophage antibody-dependent cytotoxicity by the stimulation IFN-γ and inducible nitric oxide synthase (iNOS) expression in macrophages [83, 84].

Because the suppression of IgE production is important to the early immune response, it has been suggested that IFN-α secretion by an APC controls crucial immune responses of B and T cells [69]. However, this is thought to be an indirect effect because maximal Ig response to IFN-α treatment was observed in B cells only when they interact with Th cells [78]. Other IFN-α-regulated B-cell effects include the induction of IL-15 mRNA expression in macrophages, which stimulate T cells indirectly via IL-15 activity [36, 73]; the enhancement of murine macrophage phagocytosis in combination with M-CSF and IL-4 [85]; and the regulation of hematopoietic differentiation of macrophage lineage DCs with potent APC activity [35].

IFN-α has also been shown to influence lymphocyte trafficking through autocrine effects by contributing to the mobilization of the adaptive-immune response [4, 11, 72]. This is important for the anti-infective and immunoregulatory function during an immune response and is also important to recruit T-cell, B-cell, and NK cell populations from bone marrow into specialized secondary areas/tissues for antigen presentation. This localization also delivers NK cell-produced cytokines to the affected area and increases the likelihood for T- and B-cell activation in a region where there are few antigen-specific cells [21, 62]. The effect promotes an adaptive immune response during primary infection and increases the likelihood of activating low-frequency antigen-specific cells [21]. Although it remains to be determined whether this IFN-α activity is direct or indirect, the IFN-γ-induced chemokine IP-10 has also been shown to promote the migration of T cells and monocytes, suggesting an indirect IFN-α effect [32].

IFN-α signal transduction and the immune response

To date, in vitro IFN-α mechanism-of-action studies have identified multiple protein components involved in a cellular signal-transduction cascade. In addition to regulating the immune system, this signal cascade also mediates the cellular response to inappropriate, uncontrolled cellular proliferation and viral infection. Important cellular components involved with the classical IFN response include the JAK kinases, STAT transcriptional regulators, IRF transcription factors, RNase L, 2′,5′ oligo A synthetase (2′,5′ OAS), and PKR, all of which have been described thoroughly elsewhere [86]. These experiments have identified an IFN response that includes transcriptional and nontranscriptional effects [86–88]. Although viral infection is a common method to induce an IFN response, cellular stress and radiation can also stimulate IFN-α protein production or IFN-regulated genes [12, 89].

Presumably because IFN-α regulates the transcription of numerous gene products positively and negatively, including transcriptional activators and other cytokines, the effect of IFN-α treatment has often been described as pleomorphic. In fact, the regulation of other cytokines highlights the difficulty in identifying IFN-α-specific/direct effects on the host immune system. Additionally, the JAK/STAT pathways are used by many other signal-transduction-effector molecules including IFN-γ [41, 90]. Thus, it is not surprising that the direct biological consequences of IFN-α treatment have frequently been difficult to elucidate. Furthermore, a number of controversial and dogmatically defined IFN-α functions and activities could be a result of cell-differentiation state, cell type, and concentration dependence [86]. Experiments using microarray and proteomics analysis have begun to examine the complex cellular response induced by IFN-α and IFN-γ treatment [91–94]. However, much of the intracellular IFN-α mechanism of action remains to be clarified.

IFN-α as an immunotherapeutic protein

Early in vitro studies focused on the antiviral effect of IFN-α using mammalian cell lines infected with a variety of viruses {e.g., influenza, encephalomyocarditis virus (EMC), vesicular stomatitis virus (VSV), and lymphocytic choriomeningitis virus (LCMV); for review, see ref. [95]}. In addition to a direct antiviral effect, these studies have shown that IFN-α had a dramatic, inhibitory effect on cellular proliferation [96]. Because most of the cell lines used for in vitro studies were transformed, these early findings lead to research focusing on the antineoplastic properties of the IFN-α. In fact, the first FDA-approved clinical indication for IFN-α was against Hairy Cell Leukemia (HCL) [97]. Important to a discussion of mechanism of action is the fact that the antiproliferative effect of IFN-α appears to be independent of its antiviral activity, suggesting distinct mechanisms for the two activities [98–100]. However, a clear mechanism of action of immune regulation by IFN-α has yet to be elucidated because of the complexity of the immune system and malignant/viral disease progression. Recent data from clinical trials with IFN-α have revealed that there is a significant component of the immune response involved in the observed antineoplastic and antiviral activity [10, 101, 102]. Thus, although each origin of clonal neoplasm or type of virus tends to have unique aspects to its respective regulation, it is our contention that the immunologic components modulated by IFN-α therapy are critical elements in conferring therapeutic efficacy in the clinic. As a result, disease resolution or viral eradication by IFN-α may have distinct requirements that balance a direct intracellular effect and an immunomodulatory effect of IFN-α (**Fig. 3**). Studies designed to elucidate the immunomodulatory activity further are important in the future development of IFN-α as a clinical immunotherapeutic.

ANTITUMOR IMMUNOLOGY

IFN-α has a greater demonstrated, overall impact for hematological malignancies than for solid tumors, and the immunological response to IFN-α treatment has been shown to be critical to a clinical antitumor effect [101]. IFN-α has a well-known antitumor activity in mouse and human malignancies and has been shown to decrease the tumorigenicity of transplanted tumor cells [42, 103]. In fact, an immunological role for IFN-α was demonstrated first with L1210 lymphoma cells that are resistant to IFN-α treatment in vitro, and in vivo IFN-α treatment in a murine model inhibited tumor development and growth [104]. The mechanism for this effect was partially a result of CD8+ CTL activity [104]. In a clinical setting, the first licensed approval for an IFN therapeutic in the United States was as a treatment against HCL; this IFN-α indication

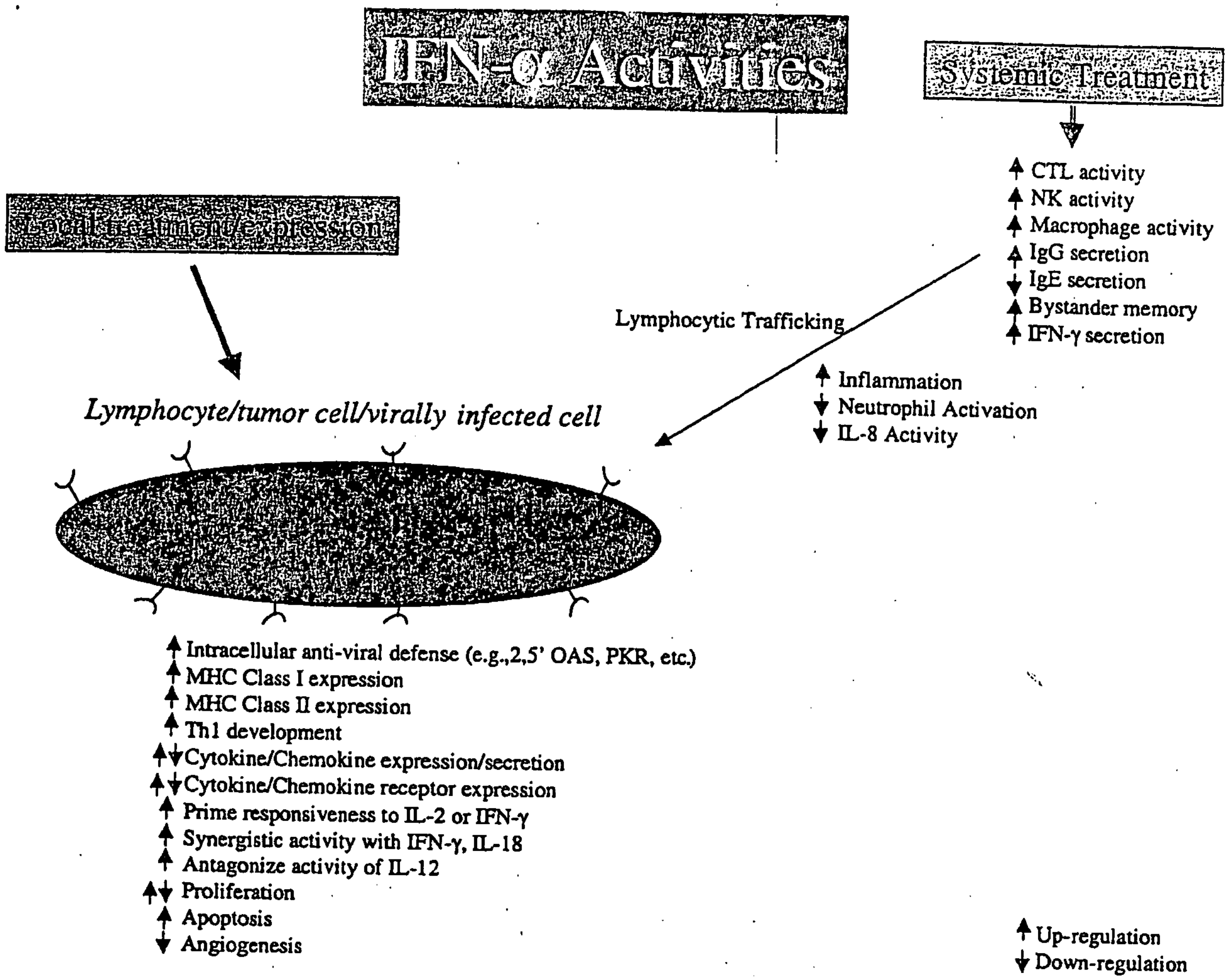

IFN-α Activities
Systemic Treatment
↑ CTL activity
↑ NK activity
↑ Macrophage activity
↑ IgG secretion
↓ IgE secretion
↑ Bystander memory
↑ IFN-γ secretion
Local treatment/expression
Lymphocytic Trafficking
↑ Inflammation
↓ Neutrophil Activation
↓ IL-8 Activity
Lymphocyte/tumor cell/virally infected cell
↑ Intracellular anti-viral defense (e.g.,2,5' OAS, PKR, etc.)
↑ MHC Class I expression
↑ MHC Class II expression
↑ Th1 development
↑↓ Cytokine/Chemokine expression/secretion
↑↓ Cytokine/Chemokine receptor expression
↑ Prime responsiveness to IL-2 or IFN-γ
↑ Synergistic activity with IFN-γ, IL-18
↑ Antagonize activity of IL-12
↑↓ Proliferation
↑ Apoptosis
↓ Angiogenesis
↑ Up-regulation
↓ Down-regulation

Fig. 3. As a priming cytokine, localized expression of IFN-α or low-dose treatment with IFN-α can result in a pleomorphic list of activities that range from an increase in apoptosis and immune-surveillance capability to decreased angiogenesis. As a regulator of multiple immune cells, systemic treatment with IFN-α can affect the balance of Th1 and Th2 cell populations. These activities are important to antiviral host defenses and antineoplastic disease responses. Complex intracellular signal-transduction mechanisms control these activities and result in such responses as the regulation of gene expression and cell proliferation.

was also the first FDA-approved biological therapy for a human malignancy [97]. During IFN-α treatment of HCL patients, a decreased bone marrow infiltration by malignant cells was observed and resulted in a normalization of peripheral hematologic variables and a decrease in patient morbidity [97].

Because IFNs elicit a pleomorphic effect and target the transcription of a specific subset of genes, their use and mechanism of action are unique compared with conventional, systemic chemotherapy that typically target DNA synthesis [101]. This makes combination treatment of IFN-α with standard chemotherapeutics more likely in future clinical trials, with the possibility of increasing antitumor activity compared with single-drug treatment [101]. Recent studies combining IFN-α with standard chemotherapeutics have begun to show the promise of such combination therapies [105, 106]. However, it is still unclear if the antitumor mechanism of action of IFN-α is because of direct antiproliferative or apoptosis effects, immune-system stimulation of a CTL-mediated immune response, or cytokine stimulation. Of course, a combination of these activities is likely. Future studies with IFN-α in human and animal models will be needed to address this gap in understanding.

What is known currently is that an important step to the elimination of a solid tumor is through immunosurveillance, and IFN-α plays an important role in controlling tumor growth through the regulation and proliferation of memory CD8+ T cells in a way that is not seen with IFN-γ, G-CSF, or IL-4 treatment [42]. Alterations in MHC-antigen expression are crucial to oncogenesis and metastasis development. Most tumor cells exhibit a partial or complete loss of MHC antigens on the cell surface [107, 108]. These tumor-specific effects result in a paucity of tumor antigen presented by APC DCs as a result of an absence of T-cell activity and the poor infiltration of DCs into tumors [108]. DCs play an important role in antitumor immunosurveillance as rare, potent APCs and IFN-α-producing cells [109]. The expression of nonself antigens when presented through class I MHC complexes is critical to the stimulation of an adaptive-immune response involving the CD8+ cytotoxic lymphocyte activity. Thus, although CTLs are crucial effectors to immunosurveillance, they are not normally activated effectively in tumor-bearing hosts. In fact, cancer patients show low or undetectable levels of CTL in peripheral blood, and reduced immunological cell performance is a prognostic indicator of antitumor-immune response for advanced cervical cancer [108, 110]. Antitumor therapies that up-regulate MHC gene expression in tumor cells (including IFNs, retinoic acid, and calcitrol) are thought to induce immunologic rejection of the tumor cell. Because IFNs can also stimulate the proliferation, activation, and generation of CTLs, this also increases the likelihood of tumor-cell cytolysis [20, 70].

Although an effective antitumor response will promote persistence of CD8+ CTL activity in the midst of a productive CD4+ activity, it is very clear that CD8+ CTL activity alone cannot reduce tumor burden [108]. In addition to CD8+ CTL antitumor activity, macrophages and NK cells have important antitumor cytotoxicity [64]. In fact, tumor cells suppress the local immune response by expressing inhibitory NK cell receptors. These inhibitory receptors negatively regulate the lytic activity of tumor-specific CTLs and demonstrate the importance of NK cell activity in reducing tumor burden [111, 112].

The development of metastasis may be under the control of cytokines and chemokines released by the tumor [113–115]. Cancer cells attempt to circumvent cytokine signaling and thus evade antitumor, antigen-specific, immune cells. There is also an inverse correlation between IFN-γ expression in tumor-infiltrating lymphocytes and disease state [115]. Recent evidence suggests that IFN-γ and perforin are critical components of tumor-suppressor response of the immune system to protect against the development of carcinogen-induced sarcomas in athymic nude mice [116]. Regulation of tumor-cell immunogenicity is key to tumor evasion of an antitumor response with documented evidence of altered IFN signal-transduction-response pathways in tumorigenic cell lines [117–119]. Together, these data underscore the importance of IFNs in antitumor activity by the host immune system.

IFN regulation of the host immune system is important to its mechanism as an antitumor activity. This influence can be seen with IFN-α treatment of tumor cells, which markedly increase the transcription and expression of class I MHC antigens [107]. IFN-γ can also increase the expression of some tumor-associated antigens and has been shown to up-regulate a variety of cell-surface adhesion molecules on tumor cells and effector cells [108, 120, 121]. As with the antiviral activity of IFN-α, IFN-α therapy promotes Th1/Th2 balance in proliferative diseases that induce a Th2-favored imbalance [29, 49]. Finally, the absence of CTL activity typically observed in patients with post-transplant lymphoproliferative disorders can be restored with IFN-α therapy [122].

The antiviral effects of IFN-α treatment in Hepatitis B and Hepatitis C disease also suppress hepatocarcinoma and liver fibrosis even when complete viral-infection eradication is not achieved [123–126]. Specifically, IFN-α HCV clinical trials show a reduced incidence of morbidity or a reduction in cirrhosis complications [127, 128]. One possible IFN-α antitumor mechanism is the induction of cell-cycle arrest in primary hepatocytes in a time- and dose-dependent manner [120, 129, 130]. This cell-cycle arrest is presumably a result of the IFN-α-induced inhibition of cyclin A and B induction, which inhibits CDK2 activity [130]. IFN-α also inhibits the proliferation of hematopoietic progenitor cells from normal and CML bone marrow including myeloid, erythroid, megakaryocyte, and multilineage colony-forming cells [120]. Separation of antiproliferative and antiviral activities via target-gene responses hints at the complexity of IFN-α-induced cellular responses in antitumor and antiviral mechanisms [98–100].

Clearance of tumorigenic cells can occur not only by a direct adaptive response through CTL activity but also through a direct induction of apoptosis [131]. IFN-α has demonstrated positive and negative effects on apoptosis, highlighting a cell type, state of cell differentiation, and context dependency for the response [86, 131]. In fact, IFN-α may play a role in inducing apoptosis in hematopoietic progenitor cells undergoing uncontrolled cellular proliferation [131]. Recent data suggest that IFN-α and IFN-γ sensitize cells to apoptosis through the up-regulation of tumor cell-surface expression of the Trail and Fas [132–136]. IFN-γ modulation of Fas expression makes carcinoma cells sensitive to antigen-specific CD8+ CTL attack [136].

The establishment of a clonal neoplasm results in the unrestricted growth of the clonal mass associated with the induction of angiogenesis to provide sufficient blood flow and nutriture to the growing mass. IFNs are important for the control of tumor inflammation and inhibit immunologically induced angiogenesis, independent of the IFN antiproliferative effects [137–140]. IFN-α-induced inhibition of angiogenesis is correlated with a decrease in β-fibroblast growth factor (BFGF) and matrix metalloprotease-9 expression and blood-vessel density. These effects lead to a cessation of progressive tumor growth [141, 142]. In fact, recent studies with a mouse-bladder cancer model have suggested that daily injections of IFN-α at a low dose show a significant antitumor response in comparison with high-dose injection therapy three times per week [142]. These studies have also demonstrated that expression of IFN-β within a tumor is inversely correlated with the level of proangiogenic molecules and vascular density [114, 141, 143]. Furthermore, the IFN-regulated chemokine IP-10 is a very potent inhibitor of angiogenesis, suggesting that IFN-α regulation of angiogenesis is mediated by an indirect mechanism [138].

By potentiating and supporting the host's immunological response against neoplastic disorder or viral infection, IFN-α therapy may enhance self-vaccination. This effect occurs through the stimulation of CTL activity and an increase of nonself antigen presentation [101]. IFN-α has been suggested for use as an adjuvant to antitumor and antiviral vaccines and for use in post-surgical, high-risk disease [42, 70, 102, 144, 145]. It is thought that the IFN-α inhibition of early T-cell activation events is important to effectively suppress adjuvant arthritis in rats and down-regulate delayed-type hypersensitivity and lymph node proliferation induced by allergen presentation [26]. Furthermore, IFN-α activity is similar to that seen with the mycobacterium Bacillus Calmette-Guerin (BCG) vaccination. Both treatments show a strong effect on macrophage activity, and they activate T-cell responses and induce IFN-γ, TNF-α, and IL-1 protein expression/secretion [146–150]. BCG vaccination efficacy is not a result of a markedly local inflammatory response; it also involves a systemic immune response involving Th cell and mononuclear cells and, in fact, may be mediated by the direct up-regulation of IFN-α [151].

CML

CML malignancy is derived from an abnormal tyrosine-kinase activity that is thought to protect CML cells from apoptotic death [101]. IFN-α uses immunological mechanisms to down-regulate CML cell growth [120, 152]. Prolonged patient survival times are observed with IFN-α treatment correlating with a normalization of hematologic indicators. CML treatment with IFN-α yields a complete hematological response in 80% of all patients [120]. Approximately 20% of all CML patients treated with IFN-α see a reduction of cells bearing the 9–22 chromosomal translocation over the course of a 12- to 24-month therapy regimen, demonstrating a complete cytogenetic response [101]. The up-regulation of NK cell activity with IFN-α treatment is correlated with clinical remission of CML, suggesting a direct immunological effect of IFN-α on the host immune system for clinical efficacy [58]. Recently, direct evidence of a role for T-cell immunity in clearance of malignant cells has been shown, correlating the presence of CML peptide-specific T cells and clinical response after IFN therapy [153]. Similar effects were seen with allogenic bone marrow-transplant therapy but not chemotherapy. These results suggest that IFN-α can induce CML remission by facilitating autologous leukemia-reactive CTL expansion [153, 154].

Because CML progenitors are unresponsive to β1-integrin-mediated inhibition of proliferation, it has been suggested that IFN-α treatment may actually reverse this responsiveness and restore the integrin regulation of cell growth and proliferation [155]. Similar effects have been seen with treatment of naïve T cells and lymphocyte proliferation in lymphoproliferative disorders where IFN-α directly inhibits [5, 120, 156]. Thus, the IFN-α mechanism of action in CML therapy minimally involves antiproliferative and immunological components and may also explain the efficacy against other lymphoproliferative disorders.

Melanoma

Maximally tolerated doses of IFN-α have been used in melanoma patients at high risk of reoccurrence after surgery [101]. In 20% of all IFN-α-treated patients, there is tumor-burden regression and reduction of reoccurrence rate [101, 157]. Preliminary data correlate therapy relapse with melanoma cell expression of nonclassical human leukocyte antigen (HLA) molecules (HLA-G) prior to IFN therapy [158]. These data suggest that IFN-α-therapy unresponsiveness might be a result of altered NK cell immunosurveillance. Additional work needs to be performed to further understand cancer patient responsiveness to IFN therapy.

In murine melanoma models and clinical trials, IFN-α therapy shows a greater antitumor activity when the tumor burden is reduced [101, 159]. Thus, the antitumor activity of IFN-α against melanoma seems to be a dose-intensive effect [101]. IFN-α alone and as an adjuvant in combination treatment with GM2 vaccine showed improvement in relapse-free survival of patients [160, 161]. These effects might be related to the adjuvant activities of IFN-α. The success of IFN-α against melanoma has led to suggestions of combination use with more traditional chemotherapeutic agents [102]. Future clinical trials will determine whether IFN-α combination therapy proves more efficacious than single-drug therapy.

ANTIVIRAL IMMUNOLOGY

IFN-α plays a very important role in the host antiviral defense by directly inhibiting the intracellular-viral lifecycle or by regulating the immune-system T-cell response during viral infection [19, 162]. IFN-α, -β, and -γ, together and separately, inhibit most stages of replication and the lifecycle of a wide variety of viruses. For example, SV40 and retroviral entry/uncoating are inhibited by IFN treatment, while influenza, VSV, and picornaviruses are inhibited by disruption of viral RNA transcription and decreased vRNA stability. Adenovirus, reovirus, and vaccinia virus have been shown to be inhibited by IFNs at the stage of viral protein translation, while retroviruses and VSV virus replication are inhibited by a block in viral particle maturation and release [86]. IFN activation of the 2′,5′ OAS and PKR proteins inhibits the cellular protein-synthesis machinery that may play an important role in inhibiting viral replication or tumor growth (for review, see refs. [21, 86]). Most importantly, although in vivo viral infection models aide in the elucidation of antiviral immunology, the mechanistic complexity of the IFN-α control of the host immune system demonstrates how many activation pathways must exist, through diverse target genes, to induce an antiviral state [8, 40, 163, 164].

Studies with IFN-α/βR$^{-/-}$ mice have yielded information regarding the regulation of the immune system by IFN-α to control viral infection. Although these mice have an otherwise normal immune system, they are unable to eradicate viral infection, have a markedly reduced NK cell activity response to infection, and lack an antiviral CD8+ T-cell CTL activity [8, 40]. Because in vivo expression of IFN-α induces an antiviral state that can protect permissive cells, IFN-α/βR$^{-/-}$ mice also show altered cell and tissue tropism during viral infection [165]. Notably, IFN-α/βR$^{-/-}$ mice are highly susceptible to viral infections despite an intact IFN-γ host immune-response pathway. Although lacking IFN-α/β induction of IFN-γ, IFN-α/βR$^{-/-}$ mice use IL-12 to regulate IFN-γ production alternatively, demonstrating a functional redundancy and plasticity within the immune system [40]. However, this functional redundancy of activating IFN-γ expression is unable to replace the loss of IFN-α/β antiviral activities. Thus, IFN-α and IFN-γ are nonredundant functionally and are essential for antiviral defense [8, 164]. In fact, although the IFN-α signal-transduction pathway is independent of IFN-γ activity, it is augmented by IFN-γ expression and the expanded signal-transduction network of IFN-γ activity [20, 37–39, 41, 42].

Because an effective adaptive-immune-response defense requires previous exposure of host to the virus, the virus can succeed in establishing an active infection of the host in the absence of an effective adaptive response. Many studies have demonstrated that in an in vivo infection, the initial response by innate-immune cells is to increase production of IFN-α/β to stimulate NK cell activity [34, 56, 57]. Recent experiments have shown that NK cell activation receptors are a component of in vivo resistance to viral infection and vital to innate-host response. This activity is based on the fact that NK cells use host activation/inhibition receptors to influence cytolytic activity [166].

Immunosurveillance of virally infected cells is an important component of the host adaptive-immune response. During a typical viral lifecycle, some of the viral proteins are transported to the cell surface as intact proteins or as peptide fragments that have been processed by the intracellular ubiquitin/proteosome/transporter in antigen processing (TAP) mechanism for presentation by the cell's MHC scaffolding. In addition to up-regulating class I MHC expression, IFN-α and IFN-γ can enhance the proteolytic processing and class I MHC presentation of viral antigens through the up-regulation of ubiquitin-conjugating enzymes, proteosome enzymes, and TAP transporter proteins [94, 167, 168]. Because ubiquitination is a rate-limiting step in antigen presentation, this means that IFNs can enhance antigen presentation by macrophages [94]. This is very important to host production of an antibody-specific cellular-cytotoxic response against the virally infected cell. When the viral antigen is presented in a cognate relationship on the infected host cell and when driven by an IFN-α-stimulated Thl T-cell response, there is a directed, adaptive, cell-mediated response by the CD8+ CTLs. Once an antigen-specific CD8+ T cell is generated, IFN-α can stimulate its CD8+ CTL activity directly [20, 70]. Numerous virus-infection models have demonstrated that activation of CD8+ CTL is critical for clearing viremia early in a primary infection, presumably because the CD8+ T cells are capable of lysing virally infected cells in an antigen-specific, HLA-restricted way [169]. Furthermore, in vivo exposure to IFN-α during the primary immune response to a viral infection induces a nonspecific, bystander CD8+ T-cell proliferation and promotes the survival of antigen-specific and nonspecific CD8+ memory T cells [70]. Tough et al. [70] present evidence that maintenance of memory CD8+ T cells is not through TCR stimulation but rather through intermittent contact with a variety of cytokines. The maintenance of memory CD8+ T cells in the absence of antigen presentation ensures an antigen-specific immune response during future infections.

Clearance of virally infected and tumorigenic cells as a result of IFN-α treatment can occur not only through an indirect adaptive response and the activation of a CTL response but also via the direct induction of apoptosis. Recent studies have suggested that IFN-α sensitizes cells to apoptosis through many of its transcriptionally regulated genes, including IRF-1, PKR, and 2′,5′ OAS [86, 131]. Specifically, the DNA-binding activity of IRF-1 has been shown to up-regulate the transcription of Caspase 1 in T cells in response to DNA damage [170]. Although IRF-3 is not regulated transcriptionally by IFN-α, IRF-3 is activated during viral infection by an unknown kinase and is a potent inducer of apoptosis [88]. IFN-α-regulated gene products have also been linked with Trail-, as-, p53-, c-myc-, and Bcl-2-dependent apoptosis [133, 135, 136, 171, 172]. These IFN-α-regulated, apoptotic mechanisms are thought to be important not only for the elimination of virally infected cells but also for the elimination of activated T cells, which limits a T-cell response and is a mechanism for controlling the proinflammatory response-immune system [134].

LCMV

LCMV is a noncytopathic virus that can induce a persistent/chronic infection and has taught much about antiviral immunology in animal model systems [173]. LCMV demonstrates general and virus-specific CD8+ effects, such as the ability of virus-specific CD8+ cells to cycle/regulate cytokine secretion and maintain a steady state of perforin expression [174]. Studies in murine persistent/chronic-infection models have shown that in the absence of IFN-α, there is no CTL response to LCMV-infected cells, which results in unregulated LCMV infection [40]. LCMV studies have also demonstrated that complete exhaustion of T-cell immunity allows for the persistence of viral infection and the depletion or silencing of viral- and antigen-specific cytotoxic T cells [175–177]. Such studies show a selective deletion of epitope-specific memory CTLs following infection with heterologous/unrelated viruses [178]. These experiments have demonstrated that LCMV infection is a model system where memory T-cell populations for multiple pathogens are accommodated over the course of a host's lifetime. It is clear from in vivo studies using LCMV that the balance of CD4+ and CD8+ cell populations within a host is important [179]. In these studies, the absence of CD4+ cells results in virus-specific CD8+ effector cells that did not secrete IFN-γ and were unable to kill virally infected cells [177, 179]. This has a clear impact on the host's ability to respond to viral infection, and these findings are similar to CD4+/CD8+ T-cell codependency data derived from CD4$^{-/-}$ mice [180]. Furthermore, LCMV studies with IFN-α/βR$^{-/-}$ mice have demonstrated that IL-12 stimulation of IFN-γ in the absence of IFN-α is not enough to clear the viral infection [40]. Thus, although the antiviral activity of IFN-α has been shown to be synergistic with IFN-γ, together IFN-α and IFN-γ are critical components of the host immune response [78, 164].

HCV

IFN-α has been the most successful antiviral/immunological therapy for the eradication of HCV infection. A comprehensive review of HCV and the disease induced by infection can be found in ref. [181]. Furthermore, thorough reviews of HCV infection regarding genotype-specific differences and patient responders versus nonresponders will not be discussed in this review but can be found elsewhere [182, 183]. Pertinent to this review, there are immune-related dysfunctions in addition to the liver cirrhosis associated with chronic HCV infection. The immune-system dysfunction is specific to the phase of HCV infection/disease. For instance, a strong Thl response and subsequent weak or absent Th2 response are observed in patients with acute HCV infection. This is in stark contrast to patients that develop a chronic HCV infection and show a predominant Th2 response correlated with weak Thl activity [183]. During chronic HCV infection, CTL activity lyses HCV-infected cells, and there is a correlation between the presence of intralobular CD8+ T cells and high-serum alanine aminotransferase (ALT) levels [184].

Important to this discussion, there is a marked decrease in HCV RNA levels in patient sera after IFN-α treatment [128]. The earliest possible treatment with IFN-α is important to the dose-dependent viral-load reduction and term efficacy [185].

Recent clinical trials have revealed important IFN-α immunoregulatory effects, including a restoration of Th1/Th2 homeostasis, decreased liver necrosis, decreased inflammation, and a normalization of ALT serum levels associated with IFN-α treatment of HCV-infected individuals [128, 183]. IFN-α therapy also counteracts the proinflammatory response by increasing the circulating concentration of soluble IL-1R and thus, inhibiting IL-1 activity [45]. Other immunological effects of IFN-α treatment are the increase of macrophage- and lymphocyte-activation markers (such as CD69) after IFN-α treatment and an enhancement of NK cell cytotoxicity, which is correlated with clinical and pathological regression of chronic HCV infection [59, 162]. Clinical trial data support the hypothesis that the success of IFN-α therapy is to potentate the host's pre-existing, antiviral response, which in the absence of IFN-α, is insufficient to eradicate the viral infection [61, 186].

Although IFN-α treatment has demonstrated a decrease in HCV RNA load in a portion of the patient population, significant clinical effects have been observed in patients with chronic hepatitis C infection treated with a combination of IFN-α plus Ribavirin [183, 187], a broad-spectrum, antiviral, ribonucleoside analogue that interferes with viral transcription, inhibits ribonucleoprotein synthesis, and has been suggested to be an RNA virus mutagen [187, 188]. Clinically, the combination of IFN-α and Ribavirin is synergistic and over 24 or 48 weeks of therapy, gives overall, sustained virology response rates of 33 and 41%, respectively [183, 187]. Mechanistically, it has been suggested that IFN-α and Ribavirin treatment in HCV chronically infected patients up-regulates IL-10 and down-regulates IL-2 and IL-12 to inhibit or reduce a cytolytic inflammatory response [187]. In comparison to single-drug therapy, the combination with IFN-α and Ribavirin also appears to restore the balance between the Th1 and Th2 cell populations more quickly and improve the efficiency of a cytolytic T-cell response in HCV-infected cells [183, 187].

The pegylated modification of IFN-α (PegIFNα) results in a protein that has a longer protein half-life in patient sera and shows equivalent activity compared with the unmodified IFN-α [189]. Clinically, the antiviral activity of combined PegIFNα/Ribavirin treatment was shown to be dose-related and synergistic compared with PegIFNα monotherapy [190]. Compared with the administration of IFN-α three times weekly, once-weekly administration of PegIFNα reduced renal clearance and improved the combination efficacy associated with reducing viral load and cirrhosis complications [191, 192]. PegIFNα clinical trials suggest that key antiviral and immunological mechanisms can be modulated by prolonged exposure to IFN-α, effectively reducing the drug-dosing regimen. In comparison with IFN-α, PegIFNα therapy also demonstrates an improved Th2 down-regulation, increased macrophage activity, down-regulation of CD4/CD8 activation after viral challenge, and improved HCV antiviral efficacy [190–192].

Inhibition of HCV replication in tissue-culture conditions, where there is no immune-system response, has been shown recently [193]. One of the primary IFN-α antiviral activities that attempts to inhibit HCV replication and translation initiation is the PKR protein. As a viral defense against intracellular IFN activity, the HCV viral proteins NS5A and E2 inhibit PKR activity [194–197]. Presumably, IFN-α also inhibits HCV replication in a manner similar to that observed with hepatitis B virus (HBV)-replication inhibition, where IFN-α inhibits the production of progeny viruses by blocking the reverse-transcriptase activity of the HBV polymerase protein [86, 198]. However, because an HCV-in vitro replication system has only been developed recently, much of the mechanism of action of IFN remains to be elucidated [199, 200].

Human immunodeficiency virus (HIV)

Recently, IFN-α therapy has been suggested as a treatment for HIV-infected individuals. In this situation, IFN-α is unique in its mechanism of action when compared with highly active, antiretroviral therapy (HAART) [201]. As an antiviral and immunotherapeutic drug, it is suggested that IFN-α can boost the host's immune system in response to HIV infection. Important to its viral tropism is the fact that HIV can replicate in quiescent and stimulated cells. Because the primary host receptor for HIV is the CD4 protein, macrophages, CD4+ T cells, DCs, and monocytes are infected initially or during reinfection from a reservoir [202–208]. Given the viral tropism for CD4+ T cells, it is not surprising that numerous immunological abnormalities occur during the asymptomatic period of HIV infection [209–212]. Because pDC2s represent the "professional" IFN-producing cells and have potent APC activity important in host defense, preservation of these cells is associated with disease protection [15, 16]. Recent data presented by Soumelis et al. [213] demonstrate a negative correlation between the number of circulating, natural IFN-α-producing cells (pDC2s) in patient blood and HIV viral load. This is the first study demonstrating that pDC2s are affected during HIV infection. pDC2 blood measurements also suggest that these cells play an important role in the protection against opportunistic infections and Kaposi sarcoma. Thus, a measure of patient pDC2 blood levels could prove to be an important parameter to monitor in assessing the status of the immune system in HIV-infected patients. During HIV infection, there is a decline in the CD4+ and CD8+ T-cell populations, decreased CD4+ Th-cell activity, failed Ig response in B cells, and decreased IFN-γ production by the host immune system [209, 214–216]. The progressive loss of CD4+ T cells is presumably because of direct HIV infection and subsequent cell deletion [210, 217]. Some studies have shown that CXCR4+/CD8+ T-cell death is macrophage-dependent and enhanced during HIV infection by the direct stimulation of macrophage cytotoxicity by the HIV gp120 protein [218]. However, the mechanisms regulating the premature turnover of CD4+ and CD8+ T cells are still not well-elucidated.

Important to this discussion is the fact that long-term nonprogressors maintain an antiviral HIV Th-cell response during infection. In HIV-infected individuals, the disruption of the adaptive immune response correlates with an increase in viral load, a decline in CD8+ CTL activity, and AIDS progression [219–222]. Recent studies have shown that there is a concomitant increase in circulating IL-7 levels, which is indicative of a homeostatic increase of IL-7 by DCs in response to T-cell depletion [223]. As has been seen in murine LCMV models, the loss of the CD4+ T-cell population negatively impacts the ability of the host to clear the viremia [48, 224, 225]. Furthermore, Champagne et al. [212] have shown that there is a skewed maturation of HIV-specific CD8+ T cells during HIV infection with an accumulation of preterminally differentiated memory T cells, possibly as a result of the rapid turnover of terminally differentiated CD8+ T cells. Apparently, this effect is HIV-specific, because it was not seen in CMV-infected patients.

As was demonstrated with LCMV studies, CD8+ T cells play a critical role in controlling HIV viremia [220, 226–229]. This is supported by in vivo animal experiments that show that in $CD4^{-/-}$ mice, the CD8+ T cells have normal cytotoxic activity but there is a greatly reduced development of class II MHC-restricted Th-cell activity [180]. In fact, two antiviral activities of CD8+ T cells have been described during HIV infection. The first mechanism involves direct cytolysis of HIV-infected cells in an antigen-specific, HLA-restrictive manner [220, 230]. The second mechanism involves the secretion of soluble factors [including the chemokines macrophage-inflammatory protein (MIP)-1α, MIP-1β, and regulated on activation, normal T expressed and secreted (RANTES)], which antagonize HIV binding to the CCR5 coreceptor [231, 232]. CD8+ T-cell secretion of CCR5 agonists inhibits R5-HIV binding to its host coreceptor, CCR5, and suppresses post-entry viral replication by down-regulating transcription in HIV-infected cells. This inhibition occurs without inducing cell death [47, 222, 224, 226, 229, 233–237].

During HIV infection, the host immune system makes virus-specific CTLs from epitopes within the Env, Gag, Pol, Nef, Tet, Tat, and Rev HIV proteins [220, 221, 230, 238, 239]. Rosenberg et al. [224] have demonstrated that the systemic control of HIV viral load was associated with an HIV-specific CD4+ T-cell response to the p24 protein. As the HIV infection progresses, there is a deletion or silencing of CTL populations specific for the gag, p24, and NP epitopes [221, 239]. Furthermore, rapid AIDS progressors elicit only a transient Gag-specific CTL response that correlates to an apparent inability to control viral replication and spread [239]. Although these "unresponsive" CD8+ CTL cells have TCR signal recognition, they do not proliferate in vitro [240]. These effects result in decreased HIV-antigen exposure or recognition and thus, decreased immunosurveillance capability.

It has been firmly established that HIV binding and entry into a host cell are dependent on the CD4 receptor and the chemokine coreceptors CCR5 and CXCR4 [231, 241]. However, given the dramatic loss of CD8+ T cells during HIV infection, a direct infection of CD8+ T cells by HIV has been suggested [206, 242]. Recent analysis of chronically infected patient sera has detected HIV isolates that infect T lymphocytes independent of CD4 binding, suggesting that their primary host receptor is actually the CD8 protein [243, 244]. The significance of this finding has to be more thoroughly examined to determine the percentage of CD8-specific HIV isolates in the patient population. Although a direct HIV infection might explain the loss or depletion of CD8+ T cells observed in HIV patients, there is also strong evidence that the primary method for reduction of the CD8+ population is the macrophage-mediated apoptosis of HIV-infected and uninfected CD8+ T cells [218]. These literature inconsistencies remain to be clarified.

There are many ways that HIV-1 infection attempts to control the host immune system and block IFN intracellular, antiviral activity. Similar to scenarios seen with HCV infection, the HIV-1 Tat protein has demonstrated anti-IFN activity by inhibiting PKR activity directly [245, 246]. Meanwhile the HIV-1 Nef protein down-regulates class I MHC expression at the cell surface by delaying transport from the endoplasmic reticulum to the plasma membrane. This effect down-regulates antigen presentation by the HIV-infected cell [247]. Furthermore, like most retroviruses that down-regulate expression of their respective receptor, HIV also down-regulates the cell-surface expression of the CD4 protein through the activity of the viral Nef protein [248]. This may have a significant impact on the normal host cell, signal-transduction cascades.

This emphasizes the potential, positive influence of IFN-α treatment in HIV-infected individuals, given its proven regulation of the adaptive-immune response. In vitro studies with IFN-α treatment of HIV-infected cells have shown a suppression of virion production during early stages of viral replication [249–254]. Type 1 IFNs also been shown to down-regulate CCR5 and CXCR4 chemokine-receptor expression on T-cell surfaces [46, 47]. Finally, IFN-α is thought to protect a cell from viral infection and protect viral antigen-specific T-cell clones. In fact, in vitro experiments with HIV-1-specific T-cell clones, which are eliminated typically during HIV-1 infection, are protected with IFN-α treatment [255]. In theory, in vivo treatment with IFN-α can reverse or overcome most of these HIV infection-specific, negative effects on the host immune response. As has been seen with HAART, decreasing viral load can reverse HIV-driven, CD4+ T-cell defects in AIDS patients [201]. In fact, recent phase II clinical trials with PEG-Intron have shown a 0.5-log decrease in viral titer during Peg-Intron treatment (unpublished results). It will be important to determine if in an HIV clinical trial, IFN-α counteracts proinflammatory cytokines such as IL-1, as was seen with IFN-α therapy in chronic HCV-infected individuals [45]. Thus, future clinical trials with IFN-α treatment of HIV-infected individuals may expand our knowledge of IFN-α regulation of the immune system in immunocompetent and immunocompromised patients.

CONCLUSION

The IFN-α mechanism of action as an antineoplastic and antiviral therapy has been elucidated slowly in the past 20 years because of the inherent complexity of the immune-system response to neoplastic and viral disease. Given these difficulties, it is understandable that the first-identified, IFN-α activities were direct, intracellular effects involving the inhibition of viral replication. However, even the direct antiproliferative/proapoptotic IFN-α activity is important to immune-cell function and plays a role in regulating the host immune response to disease. Furthermore, IFN-α acts directly and indirectly on many cell functions in the immune response to neoplasm and viral infection, including the induction of important downstream cytokines such as IFN-γ. As an early response cytokine, IFN-α is poised as a key priming cytokine for the immune antineoplastic and antiviral response. Local effects of

IFN-α expression include the activation of an immediate and effective innate-immune response. IFN-α then plays a critical role in directing the transition from innate to adaptive immunity through a variety of mechanisms including the control of host Th1/Th2 responses and the regulation of CD8+ CTL activity and memory. The expanding body of preclinical experience with IFN-α suggests that immunomodulation plays a significant role in mediating the therapeutic effects exhibited in clinical trials. However, to date, an explanation for the mechanism of action of IFN-α in various disease states remains theoretical but clearly deserving of further study. Given the complexity of IFN-α regulation cited herein, much of the work will have to come from animal studies in which the immune system can be manipulated. Unfortunately, many of the diseases for which IFN-α is indicated are not modeled easily in animals. Thus, additional disease models and future clinical trials will be needed to elucidate the role of IFN-α in immune-system regulation.

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Review Article

Imiquimod applied topically: a novel immune response modifier and new class of drug

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Abstract

Imiquimod (S-26308, R-837) (1-(2-methylpropyl)-1*H*-imidazo[4,5-c]quinolin-4 amine), an immune response modifier, demonstrates potent antiviral and antitumor activity in animal models (see structure in Fig. 1). The drug exhibits no direct antiviral or antiproliferative activity when tested in a number of cell culture systems. Imiquimod's activity was discovered while screening for anti-herpes virus activity. One of the first analogs in the series, S-25059 was tested in the early 1980's and due to slight toxicity, caused slightly reduced herpes cytopathology in Vero cell cultures. Follow-up testing in herpes infected guinea pigs showed complete protection toward lesion development. Activity of these drugs results primarily from interferon alpha (IFN-α) induction and other cytokine induction. At least part of the cytokine induction is mediated through NF-κB activation. These cytokines stimulate several other aspects of the innate immune response. In addition, imiquimod stimulates acquired immunity, in particular the cellular arm which is important for control of viral infections and various tumors. This effect is mediated by drug induced IFN-α and Interleukin-12 (IL-12) and IFN-γ induced by these cytokines. Imiquimod is expected to be effective where exogenous IFN-α has shown utility and where enhancement of cell-mediated immunity is needed. The following is a brief review of the preclinical pharmacology of imiquimod and the clinical results of genital wart trials. The mechanism of action of topically applied imiquimod will likely lead to benefits in several other chronic virus infections and tumors of the skin. Two other reviews on imiquimod that focus mainly on the clinical results have been published (Beutner & Geisse, 1997; Slade, Owens, Tomai & Miller, 1998). © 1999 International Society for Immunopharmacology. Published by Elsevier Science Ltd.

Key words: Cell mediated immunity; Condylomata acuminata; Cytokines; Human papillomavirus; Imiquimod; Immune response modifier; Interferon; Mechanism of action; Monocytes; Preclinical; Review; Therapy

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1. Effects on innate immunity

When incubated with mouse spleen cells in vitro, imiquimod at 0.2 μg/ml induces the synthesis and release of IFN, IL-6, tumor necrosis factor-α (TNF-α) and probably other cytokines (Reiter, Testerman, Miller, Weeks & Tomai, 1994). Imiquimod also causes non-specific B-cell proliferation which may be mediated directly by the drug and not through cytokine induction (Tomai, Imbertson, Wagner, Reiter & Miller, 1994).

The mouse macrophage cell line, RAW 264.7, produces TNF in response to 3 μg/ml of imiquimod. Saturable specific binding of closely related analogs of imiquimod to membrane fractions from these cells suggests the presence of a membrane receptor for these drugs (Miller et al., 1995).

When administered orally or parenterally to mice, imiquimod induces increased serum concentrations of IFN-α, TNF-α and IL-6 between 1 and 4 h after dosing (Reiter et al., 1994). Effective oral doses range from 1–250 mg/kg. Multiple doses of imiquimod on the same day cause augmented IFN-α levels, however high daily doses of imiquimod to mice results in a hyporesponsive state characterized by reduced cytokine induction. Separation of the doses by four or more days causes normal levels of cytokine induction. Oral imiquimod also causes increased levels of serum 2′,5′ oligoadenylate synthetase (2′,5′ AS) (Miller et al., 1994) which is an IFN inducible enzyme believed to be partly responsible for IFN's antiviral properties (DeBenedetti, Pytel & Baglioni, 1987). A single drug treatment causes elevated 2′,5′ AS levels for 3–4 days which supports 2–3 times per week dosing in efficacy studies (Miller et al., 1994). The use of knock-out mice indicate that STAT-1 is needed for priming and maximal production of IFN in mice treated with imiquimod (Bottrel, Levy, Tomai & Reis, 1997).

Topical application of the 1% or 5% cream formulation of imiquimod to the skin of hairless mice induces increased IFN-α messenger RNA (mRNA) levels and increased protein concentrations of IFN and TNF-α in the skin at the treatment site (Tomai et al., 1997; Imbertson et al., 1998). Cytokine increases are seen from 1–4 h after application and are not seen in skin taken from the untreated side of the mice or the site where placebo cream was applied. Topical treatment of hairless mice with imiquimod causes Langerhans cells in the skin to enlarge, appear activated and migrate from the treatment site to the regional lymph node (Suzuki et al., 1998). These activated Langerhans cells may enhance antigen presentation to T cells.

Intravaginal application of imiquimod cream to mice induces increases in vaginal tissue con-

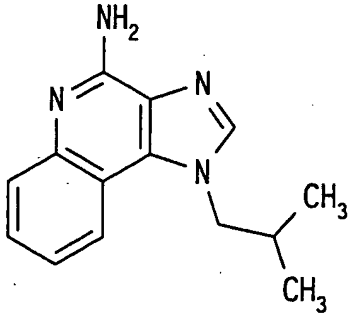

Fig. 1. 5-26308, R 837, Imiquimod.

centrations of IFN, TNF-α and 2′,5′-AS. Vaginal washes also contained increased 2′,5′-AS concentrations. Serum levels of IFN, TNF-α and 2′,5′-AS are increased after intravaginal drug application. The serum cytokine levels and kinetics are similar to an equivalent dose of imiquimod given orally.

In rats, oral administration of 3 mg/kg or more of imiquimod induces increased serum levels of IFN-α and TNF-α. The kinetics of induction are similar to those seen in mice. Hyporesponsiveness is seen in rats after multiple high daily doses. As in mice, topical application of imiquimod cream (1% or 5%) to the skin of hairless rats leads to local induction of TNF-α at the application site (Imbertson et al., 1998). In guinea pigs, 3 mg/kg of imiquimod induces serum levels of IFN-α when the drug is administered orally, intravaginally, topically, or parenterally (Miller, Imbertson, Reiter, Pecore & Gerster, 1986). In monkeys, multiple oral imiquimod doses of 3 mg/kg induce serum levels of IFN-α, interleukin-1 receptor antagonist (IL-1RA) and, in rare instances, low levels of IL-6. Peripheral blood mononuclear cell (PBMC) cultures from monkeys produce increased levels of messenger RNA (mRNA) and cytokine for IFN, IL-1β, IL-6 and IL-8 after treatment with imiquimod in vitro (Wagner et al., 1997). Hyporesponsiveness is not seen in guinea pigs or monkeys when low doses of imiquimod are used. Generally, 2–3 mg/kg is a minimum effective oral dose for IFN-α induction in different species including humans.

In human PBMCs, specifically monocytes, imiquimod at 1–5 μg/ml induces the production of several cytokines including several subtypes of IFN-α, TNF-α, IL-1, IL-1RA, IL-6, IL-8, IL-10, IL-12 p40, granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and macrophage inflammatory protein 1-α (MIP-1), MIP-1β, and macrophage chemotactic protein (MCP-1) (Weeks & Gibson, 1995; Gibson et al., 1995). Generally, a low drug concentration (about 0.5 μg/ml) is found at which IFN-α and IL-1RA are the only cytokines increased. Cytokines are detected as early as 1–4 h after stimulation with drug and this induction requires both mRNA and protein synthesis (Testerman, Gerster, Imbertson et al., 1995; Megyeri et al., 1995). Induction of these cytokines occurs through activation of transcription factors that bind to the promoter regions of IFN-α (α4F1 complexes) and a number of the proinflammatory cytokines (Nuclear factor kappa-B (NF-κB)) and activate transcription (Megyeri et al., 1995).

Keratinocytes isolated from human skin and cells from a human epidermal cell line also respond to 1 μg/ml imiquimod by producing increases in mRNA for IFN-α, IL-6 and IL-8 but not TNF-α or IL-1 (Kono et al., 1994). Slight increases in IL-8 protein levels are seen; however, imiquimod does not increase protein concentrations of IFN, IL-1α, IL-6 and TNF-α in these cultures at 6 or 24 h (Miller et al., 1995; Kono et al., 1994).

In addition to IFN and other cytokine induction, imiquimod causes stimulation of several other aspects of the innate immune response. For example, natural killer cell activity is stimulated in mice, probably due to induction of IFN-α and other cytokines induced by imiquimod. Macrophages are activated to secrete cytokines and nitric oxide. Proliferation and differentiation of B-lymphocytes is also caused by imiquimod and appears to be a direct drug effect on these cells.

As a result of these effects on innate immune responses, imiquimod has been shown to be effective in animal models against a number of viral infections and a variety of transplantable tumors. In herpes simplex virus (HSV) infected guinea pigs, a single treatment of 2–3 mg/kg of imiquimod given orally, parenterally, intravaginally, or topically is protective against primary infection when given between 72 h before and 24 h after inoculation (Miller et al., 1985; Harrison, Jenski, Voychehovski & Bernstein, 1988). In mice, imiquimod causes an increase in survival in Rift Valley Fever virus infection and Banzi virus infection (Kende, Lupton & Canonico, 1988). In Rift Valley Fever virus infected mice, production of IFN-α is critical for the antiviral effect since antibody to murine IFN-α blocks much of the increase in survival induced by imiquimod. The duration of antiviral activity lasts for 3–4 days after each oral imiquimod administration and correlates with elevation of 2′,5′-AS activity. Elevated 2′,5′-AS has been observed in the serum of mice, rats, guinea pigs, monkeys, and humans (Tomai et al., 1997) from 24–72 h after oral treatment with imiquimod. Induction of 2′,5′-AS is indirect through the production of IFN-α since production is abrogated in IFN-α/β receptor knock-out mice (Bottrel et al., 1997). Acute antiviral activity is also seen in cytomegalovirus infection models, both in guinea pigs (Chen, Griffith, Lucia & Hsiung, 1988) and in mice. However, topical application of 5% imiquimod cream was ineffective in the human papillomavirus (HPV) type 11 infected external human-severe combined immunodeficiency (SCID) mouse model (Bonnez, DaRin, Borkhuis, Rose & Miller, 1996). Finally, in a rabbit papillomavirus infection model in rabbits, topically applied imiquimod was ineffective, which is likely due to the drug's inability to induce IFN and possibly other cytokines in this species.

Antitumor activity of imiquimod is also seen in a number of transplantable mouse tumor models (Sidky et al., 1992). When given acutely, the drug is effective at reducing tumor volumes in mice given cells from a number of lines including MC-26 colon carcinoma, B16-F10 melanoma, Lewis lung carcinoma, FCB bladder carcinoma, RIF-1 sarcoma and MBT-2 bladder cell carcinoma. Imiquimod was also effective at inhibiting growth of the human mammary tumor MCF-7 when transplanted into nude mice which lack T cells. This indicates that acutely, T cells are not required for the antitumor effects of imiquimod. Much of the antitumor effect with imiquimod is again blocked by administration of antibodies to IFN-α; however, TNF-α also seems to be involved. These results suggest that imiquimod's effects on the innate immune response, in particular its ability to induce IFN-α and other cytokines, are largely responsible for its acute antiviral and antitumor effects.

2. Effects on acquired immunity

Although imiquimod does not stimulate T cells to divide or directly induce T cell cytokines such as IL-2, IL-4 or IL-5, imiquimod is capable of indirectly stimulating production of the T helper type 1 (Th1) cytokine, IFN-γ, in mouse splenic and bone marrow cultures as well as human PBMC cultures. Production of IFN-γ in response to imiquimod is inhibited by antibodies to IL-12 and IFN-α, demonstrating the importance of these monocyte/macrophage cytokines (Tomai et al., 1998). The mechanism of interaction between these cytokines has recently been defined (Rogge et al., 1997; Szabo, Dighe, Gubler & Murphy, 1997). Results show that IFN-α induces the IL-12 receptor β2 subunit on Th1 cells. These cells can then respond to IL-12 and produce IFN-γ. Thus, Th1 cells are the major source of IFN-γ; however, cytotoxic T cells and NK cells are also able to produce IFN-γ in response to imiquimod.

The ability of imiquimod to stimulate IFN-α, IL-12 and IFN-γ, cytokines known to be involved in driving the cellular arm of the acquired immune response and imiquimod's ability when applied topically to stimulate Langerhans cells are likely important in models where imiquimod has demonstrated long-lasting protection. For example, treatment of guinea pigs after primary HSV-

2 infection with imiquimod reduces recurrences both during the treatment period and even after treatment has stopped (Harrison, Miller & Bernstein, 1994). The prolonged effect after treatment is likely due to increased cellular immunity to HSV antigens and HSV infected cells (Bernstein & Harrison 1989; Harrison, Stanberry & Bernstein, 1991; Bernstein, Miller & Harrison, 1993a; Harrison et al., 1994). In addition, imiquimod can serve as a vaccine adjuvant for a HSV glycoprotein vaccine preparation in guinea pigs when given both prophylactically and therapeutically (Bernstein, Miller & Harrison, 1993b; Bernstein, Harrison, Tepe, Shahwan & Miller, 1995). Imiquimod is more effective than complete Freund's adjuvant in this model. In mice, imiquimod also enhances rejection of tumors caused by cells which express the HPV 16 E7 gene. Imiquimod causes a reduction in the control of EL4 tumors by only 9% and a reduction of the E7 expressing tumors by 51% in sham immunized mice. In the E7 immunized mice, imiquimod has no effect on the control EL4 tumors but reduces the weight of the E7 expressing tumors by 84%, which is associated with stimulation of a delayed type hypersensitivity skin test reaction (Th1) to the E7 protein. Finally, in mice implanted with FCB bladder carcinoma cells, certain regimens of imiquimod actually lead to total eradication of the tumor. These mice are totally resistant for at least eight months to rechallenge with the same FCB tumor cells but remain sensitive to challenge with a different tumor cell (Borden, Sidky & Weeks, 1991). The long-lasting immunity observed is likely via the cell mediated arm.

Imiquimod also has been shown to inhibit production of the Th2 cytokine IL-5 in both mouse and human cell systems. Inhibition of IL-5 production is mediated by IFN-α and IFN-γ. As a result of this ability to inhibit IL-5 production, imiquimod has also been found to inhibit both antigen and sephadex induced eosinophilia in several animal models (Hammerbeck et al., 1997). In addition, imiquimod has been found to inhibit virus induced eosinophilia in rats (Stokes et al., 1998). These results suggest the possibility that imiquimod may be useful in atopic diseases as well as other diseases where an increased Th1 response is needed.

Imiquimod treatment of murine B-cells also changes the immunoglobulin (Ig) response to antigens (Tygrett, Li, Tomai & Waldschmidt, 1995). Levels of the Th1 Ig, IgG2a, are increased and levels of the Th2 Igs, IgG1 and IgE, are decreased.

In summary, imiquimod induces cytokines in skin cells and blood cells and stimulates or enhances both the innate response and the cellular immune system. Long lasting immunity is demonstrated in the recurrent HSV guinea pig model and in the FCB mouse tumor model. Increased delayed type hypersensitivity is demonstrated in the HPV mouse model. Table 1 summarizes the lowest effective concentration of imiquimod in the various models. These preclinical pharmacology results indicate the potential of imiquimod for treatment of virus infections or tumors in humans.

3. Clinical mechanism of action study

The data generated in animal models suggest that imiquimod's antiviral and antitumor effects are largely mediated through the induction of cytokines that drive the innate and cell-mediated immune response. A study was carried out in humans to further explore the drug's mechanism of action (Tyring et al., 1998). The objective of this study was to evaluate the mechanism of action of imiquimod 5% cream when applied topically to genital warts in human patients by: (1) invest-

Table 1
Lowest effective concentrations of imiquimod

Species	In vivo/in vitro	Results	Dose (mg/kg) or concentration (μg/ml) of imiquimod
Mouse	In vitro (spleen cells)	IFN, IL-6, TNF-α	0.2 μg/ml
Mouse	In vitro (spleen cells)	IL-5 inhibition	0.5 μg/ml
Mouse	In vitro (spleen cells)	IFNγ induction	0.1 μg/ml
Mouse	In vitro (spleen cells)	B-cell proliferation	0.1 μg/ml
Mouse	In vitro (RAW cells)	TNF-α induction	3 μg/ml
Mouse	In vivo (oral)	IFN, TNF-α, IL-6 induction in serum	1–3 mg/kg
Mouse	In vivo (oral)	Antiviral (RVF, Banzi, Influenza)	3 mg/kg
Mouse	In vivo (oral)	Antitumor	50 mg/kg
Mouse	In vivo (topical)	IFN, TNF-α induction	(10 μl 1%) 4 mg/kg
Rat	In vivo (oral)	IFN Induction	3 mg/kg
Rat	In vivo (topical)	TNF-α induction	(100 μl 1%) 4 mg/kg
Rat	In vivo (oral)	Hyperreactivity	3 mg/kg
Guinea Pig	In vivo (oral, intravaginal, sub cut)	Antiviral (HSV-1, HSV-2, CMV)	3 mg/kg
Guinea Pig	In vivo (oral, intravaginal, sub cut)	IFN induction	3 mg/kg
Monkey	In vitro (PBMC)	IFN-α induction	0.5 μg/ml
Monkey	In vivo (oral)	IFN-α induction	3* mg/kg
Monkey	In vivo (oral)	Antiviral (Yellow Fever Virus)	10 mg/kg
Human	In vitro (PBMC)	IFN-α, IL-1RA induction	0.5 μg/ml
Human	In vitro (PBMC)	Il-5 inhibition	0.1 μg/ml
Human	keratinocytes	IFN-α, IL-6, IL-8 mRNA induction	1.0 μg/ml
Human	In vivo (oral)	IFN, IL-1RA induction in serum	2–3 mg/kg
Human	Intravaginal	IFN, IL-1RA induction in serum	(5 g 3%) 2–3 mg/kg
Human	Topical	Antiviral (warts)	(5% 3/Week) 0.1 mg/kg

* Multiple dosing required.

igating local and systemic cytokine induction; (2) assessing cellular infiltration into the warts; and (3) evaluating effects of imiquimod on HPV DNA and gene expression. In this Phase I double-blind, randomized, parallel group study, imiquimod 5% cream or placebo was applied to warts three times a week for up to 16 weeks. Serum and biopsies of warts were taken at predose, after six weeks of treatment, and at the end of study. As an inclusion criteria, HPV infection was confirmed in the predose biopsy. Biopsies were analysed by the polymerase chain reaction (PCR) for HPV DNA (copies/cell) and by reverse transcriptase (RT)-PCR for mRNAs to a number of cytokines, cellular markers and viral gene products. Changes from baseline at six weeks and at end of treatment were compared between treatments.

Results showed that all imiquimod treated patients had a ⩾75% reduction in wart area. Safety analysis revealed that most (15/16) patients receiving imiquimod cream experienced erythema at the application site that was significantly different from the vehicle treated patients. Imiquimod treatment stimulated significant increases in IFN-α and increases in TNF-α mRNAs, cytokines previously found to be induced by imiquimod in animal studies (Miller et al., 1986; 1994; 1995;

Reiter et al., 1994; Tomai et al., 1997; Wagner et al., 1997) and in human PBMC studies (Weeks & Gibson, 1994; Gibson et al., 1995; Testerman et al., 1995). A number of IFN-α and TNF-α inducible effects were also increased in imiquimod treated patients (Mx-B, 2′,5′-AS, IFN-β). IL-12 p40 and IL-8 mRNA were also increased in many patients receiving imiquimod, but these were not statistically significant. Decreases in CD1a mRNA associated with Langerhans cells were seen in imiquimod treated patients, suggesting that these cells were activated or migrated to the draining lymph node. Cytokines associated with a Th1 immune response (IFN-γ, IL-2 and IL-12 p40) were increased in many imiquimod treated patients as were CD4 and CD8 mRNAs which indicates activation of a cell mediated immune response. Increased CD4 mRNA correlated with increases in expression of CD29 and CD45Ro mRNA which are expressed on activated cells and memory cells, respectively. Wart regression strongly correlated with a decrease in viral load as measured by a decrease in HPV-DNA and a decrease in expression of both HPV early (E7) and late (L1) mRNAs. Coincident with wart regression and diminished virus was a decrease in mRNA expression for markers associated with hyperproliferation (PCNA, c-*myc*) and an increase in markers associated with differentiation (Fillagrin, involucrin, p53 and Rb). These changes are likely a result of taking wart tissue at baseline and normal appearing skin at the wart site at the end of treatment. In conclusion, wart regression by imiquimod is associated with an induction of local cytokines and cellular infiltrates that are involved with generation of a cell mediated immune response. These results in humans are consistent with the preclinical results generated with imiquimod in animal models.

4. Summary of clinical efficacy trials

Imiquimod cream was applied topically and tested for efficacy in patients with external genital and/or perianal warts (condylomata acuminata). Genital warts, the most common viral sexually transmitted disease, was chosen as the first clinical target because injectable IFN-α had demonstrated some benefit and the current therapies do not meet the patient's or physician's needs. Patient dissatisfaction with current therapeutic options is significant due to pain, tissue destruction, high recurrence rates, expense, and time required for treatment. In addition, current treatments only treat the visible wart symptoms and do not treat the underlying HPV infection. Published results indicate that biopsies of warts from these patients show little immune recognition but biopsies from warts undergoing spontaneous regression show monocytic cellular infiltration and increased Th1 cytokine expression (Tagami, Oku & Iwatsuki, 1985; Coleman et al., 1994). Similar results are seen in patients treated with interferon (Arany & Tyring, 1996). We reasoned that an immune response modifier that stimulates cell mediated immunity should be an improved therapy for genital warts.

A Phase II study in 108 patients with genital warts compared topically applied 5% imiquimod cream to vehicle cream with 23–24 h application, three days/week for eight weeks (Beutner et al., 1998). The imiquimod group had 40% complete wart clearance compared to no complete clearance in the vehicle group. In addition, there was a median 90% reduction in wart area at the end of treatment among the imiquimod group but no change in wart area in the vehicle treated group. Patients who totally cleared their lesions entered a 10 week follow-up period to observe wart recurrence and 81% of the imiquimod treated group remained wart free. In this trial, acceptable safety and efficacy was demonstrated by 5% imiquimod cream in genital wart patients.

A Phase III multi-centered, randomized, double blind, placebo controlled trial compared the safety and efficacy of imiquimod 5% cream and 1% cream with vehicle (Edwards et al., 1998). Patients applied the cream to their warts overnight for 8 h three times per week until their warts were totally cleared or for a maximum of 16 weeks. The main outcome measurements were the number of patients experiencing the complete elimination of all baseline warts and the recurrence of these warts. In addition, the reduction in baseline wart area, the duration of therapy required to eliminate warts, and the frequency and severity of adverse reactions were monitored. Patients who totally cleared their warts were entered into a 12 week follow up period to monitor recurrence of their warts.

The three times per week trial included 180 men and 131 women 18 years or older having 2–50 external anogenital warts. In the intent to treat analysis, 50% (54/109) of the patients who received 5% imiquimod cream, 21% (21/102) of those who received 1% imiquimod cream, and 11% (11/100) of patients treated with vehicle completely cleared all their baseline warts. In the treatment failures analysis, clearance was observed in 56, 27 and 14%, respectively. The difference between the effectiveness of 5% cream and vehicle was statistically significant ($P < 0.0001$) using either method of analysis. The results using 1% cream were not significantly different from vehicle. The median time to clearance was 10 weeks, 12 weeks, and 12 weeks, respectively. Females had a higher clearance rate (77, 46 and 28%, respectively) than males (40, 10 and 6%, respectively). In addition, females had a shorter median time to clearance (8 weeks) than males (12 weeks) in both imiquimod groups. The better response in females could be due to several factors including shorter duration of warts in females (3.4 months median) vs males (6.7 months median), better compliance in females or better drug absorption in females. Of the patients whose warts completely cleared during therapy, 13% (6/45) of the patients treated with 5% cream had a recurrence of at least one wart. No recurrences (0/18) were seen in the 1% patients who cleared and recurrences were seen in 10% (1/10) of the vehicle patients who totally cleared their baseline warts. Since the initial clearance rate was highest for the 5% group, the sustained wart free period was also greatest for the 5% group.

The treatment was well tolerated. Local erythema was the most common adverse reaction (67, 26 and 24%, respectively) but the majority of patients in each group experienced no or only mild local inflammatory reactions. Less than 1.2% of the patients discontinued due to side effects. There were no differences in the incidence of flu-like symptoms among the treatment groups indicating no systemic effects from cytokine induction by the drug. System effects were not expected since results of a study using radiolabeled imiquimod showed that $<1\%$ of the radiolabeled imiquimod applied was absorbed into the systemic circulation (Owens et al., 1997). In addition, a 21 day cumulative irritation study demonstrated that imiquimod cream 5% was less irritating than Vaseline® Intensive Care Lotion®, the reference cream used in the study (Owens et al., 1997).

A second Phase III trial was carried out in 154 male and 125 female patients with genital warts using daily application of 5%, 1%, or vehicle with 8 h application until wart clearance or 16 weeks maximum (Beutner et al., 1996; Beutner et al, 1998a). In this trial, 71% (49/94) of the 5% patients, 16% (13/90) of the 1% patients and 4% (3/95) of the vehicle patients had complete clearing of their baseline warts, $P < 0.0001$ when comparing the 5% and vehicle groups. Clearance in the 1% group and the vehicle group were not significantly different. Recurrence rates were 19% (9/48) for

5% imiquimod group, 17% (2/12) for the 1% group, and 0% (0/3) for the vehicle group. The low recurrence rate in the vehicle groups is not surprising since the mechanism of spontaneous clearance has been shown to be due to immune recognition (Tagami et al., 1985; Coleman et al., 1994). Local skin reactions were more common and more severe with daily treatment but there were no systemic adverse reactions. The daily treatment regimen resulted in somewhat increased rate of total wart clearance when compared to three times per week application but also resulted in greater local skin reactions.

A vehicle controlled safety and efficacy trial was also carried out in HIV-positive genital wart patients (Conant et al., 1998). The primary objective of this multi-national, multi-center, double blind, vehicle controlled, parallel group trial was to evaluate the safety of imiquimod 5% cream in HIV-positive patients. A secondary objective was to access wart clearance and reduction in wart area. A total of 100 patients (97 males and 3 females) were enrolled and treated three times per week for up to 16 weeks or until wart clearance. Imiquimod was applied to 65 patients and vehicle was applied to 35 patients. No local skin reactions were seen in a majority of patients and only mild erythema was seen in most of the others. The intent to treat analysis of all patients showed that 11% of the imiquimod patients achieved complete wart clearance compared to 6% of the vehicle group, which was not significantly different. However, there was a statistically significant difference between treatment groups for patients who achieved $>50\%$ reduction in wart area; 38% for imiquimod and 14% for vehicle ($P = 0.013$). This was a clinically meaningful reduction in wart area since wart area increases are frequently seen in these patients. These results suggest that in HIV patients, imiquimod induces the innate response which stops wart growth and causes wart area reduction and may, in part, be IFN-α mediated. However, the reduced total wart clearance in HIV patients compared to immunocompetent genital wart patients suggests a role for T-cell responses in initial wart clearance as well as in long term protection from recurrence. Imiquimod has an acceptable safety profile in HIV-positive and AIDS patients.

5. Oral delivery

Some clinical testing of imiquimod was also carried out by the oral route. A single dose study in normal volunteers indicated that measurable serum IFN levels were obtained in four of six subjects after 200 mg and in six of six subjects after 300 mg. Peak levels of IFN were seen at 12 h after dosing and levels returned to baseline by 24 h. Activity of 2,5-AS was elevated for 96 h after the 300 mg dose and this activity correlated with antiviral activity in the subject PBMC's. The drug was well tolerated (Imbertson et al., 1992). Phase I multiple dose studies using imiquimod were completed in cancer patients using different dosing schedules (Witt et al. 1993; Savage, Horton, Moore, Owens, Witt & Gore, 1996). An oral Phase I dose escalating study was also carried out in asymptomatic HIV positive individuals (Goldstein et al., 1998). Both cancer patients and HIV positive individuals responded to imiquimod with serum IFN production. Despite activity when given orally, topical administration of imiquimod is the preferred route of delivery.

6. New class of drug

The preclinical and mechanism of action study in patients indicate that topically applied imiquimod results in the induction of several cytokines at the treatment site. Cytokines such as IFN and others inhibit virus production and inhibit tumor cell growth. The drug also enhances aspects of the cell mediated immune (CMI) response and may result in long term protection from the initial virus or tumor. Application of the drug to warts by wart patients, in the privacy of their own home produced a high genital wart clearance rate with limited side effects. Results were better in women, perhaps due to a shorter duration of the warts, to better compliance with treatment or enhanced drug absorption through poorly keratinized and occluded epithelium. Both three times per week and daily treatment regimens were acceptable for safety and efficacy, however in the final analysis, the three times per week regimen was preferred for most patients. Imiquimod 5% cream (ALDARA^{TM}, 3M Pharmaceuticals) received approval by the FDA in February 1997 and is currently available in the U.S.A. for the treatment of external genital and perianal warts. Approvals are expected in additional countries in the future.

The cytokines induced by topically applied imiquimod include IFN-α, TNF-α, and IL-12p40 and indirectly, IFN-β and IFN-γ. Induction of these cytokines stimulates the Th1 CMI response and the preclinical data suggest the suppression of Th2 immune responses. This mechanism of action should cause imiquimod to be an effective treatment for chronic virus infections of the skin such as Human Papillomavirus in genital warts, and theoretically in common warts, plantar warts, Herpes simplex virus infection and Molluscum contagiosum.

Skin lesions caused by other intracellular pathogens that might also respond include intracellular bacteria such as leprosy and intracellular parasites such as leishmania. Preliminary in vitro studies using imiquimod in mouse bone marrow derived macrophages showed inhibition of *Leishmania donovani* proliferation and the topical application of imiquimod cream to mice infected with *L. major* caused a reduction in the severity of lesions (Buates & Matlashewski, 1997a,b). In addition, more potent analogs of imiquimod are able to inhibit in vitro growth of Mycobacterium avium in human monocytes (Shiratsuchi, Sherman, Miller & Ellner, 1995). Further studies are needed to confirm these activities.

Other possible uses include ultraviolet induced skin lesions such as actinic keratosis and skin tumors such as basal cell carcinoma, squamous cell carcinoma, and perhaps even melanoma. Results of a small pilot trial of imiquimod 5% cream in patients with the skin cancer, Bowen's disease, showed that 14 of 16 patients cleared their lesions (MacKenzie-Wood et al., 1998) Other skin tumors that might respond include Kaposi's sarcoma and Cutaneous T-cell lymphoma.

Since Th2 responses can be inhibited in preclinical animal models by imiquimod, atopic based skin inflammation such as atopic dermatitis might also benefit. Other conditions that may respond to topically applied imiquimod include Alopecia areata, keloids and cutaneous symptoms of the Th2 mediated autoimmune disease, Systemic Lupus Erythrematosus (SLE). Another possible use for these drugs is application with a vaccine for adjuvant activity. The imidazoquinolines are expected to enhance a Th1 response to the vaccine which could be beneficial for virus or tumor vaccines. Drug application topically or transdermally could be explored with the injectable vaccine. On the other hand, skin inflammation due to excessive Th1 responses, such as psoriasis and contact dermatitis, might be worsened by topical treatment with imiquimod. Among drugs, imiquimod is unique in being a topically active cytokine inducer and stimulant for the CMI response. Overall, imiquimod applied topically is an Immune Response Modifier which should be a useful addition to the drugs that can be used to treat significant and chronic conditions of the skin. As such, imiquimod applied topically represents a new class of drug.

The Th1 CMI response is very effective in most people in controlling virus infections and tumors.

For example, Chicken pox infection is almost universal and after the outbreak, the Varicella zoster virus responsible is carried in the dorsal root ganglia for the rest of the individual's life with no further lesions. However, lesions can occur following suppression of cellular immunity. Epidemiology studies report the Human Papillomavirus is also a frequently occurring infection with 50–75% of sexually active adults having an antibody response to the virus (Koutsky, 1997). About 15% of these individuals carry the virus and if the cellular immune response is suppressed due to anti-graft rejection drugs following transplantation, anti-cancer chemotherapy, acquiring HIV infection or in some cases of pregnancy, a severe outbreak of warts can occur. These are just two examples that demonstrate the role of the Th1 CMI response in suppressing virus lesions.

One can consider why some patients develop chronic virus lesions like warts but many times more people are infected and develop an appropriate immune response and have minor or even no symptoms (Koutsky, 1997). Infected patients who have symptoms may have mounted a Th2 response to their infection rather than the Th1, CMI response needed to eliminate the infected cells. Their Th2 response to the virus may result from a dominant Th2 response to a bacterial infection that was ongoing at the time they first encountered the virus. Elevated IL-4 levels at that time would suppress the needed Th1 response (Szabo et al., 1997). On the other hand, an ongoing Th1 response to a viral infection with IFN-α and IFN-γ induction could prevent a Th2 response and cause an inappropriate and ineffective immune response to a subsequent bacterial infection. This might explain the propensity patients with viral pneumonia or influenza have toward development of bacterial pneumonia which can be severe and even cause death (Couch et al., 1986). Animals also have severe bacterial infections after viral infections. For example, shipping fever in cattle results from a Pasteurella bacterial infection developing after a respiratory herpes virus infection (Frank, 1983). If this explanation is true, early treatment with an imidazoquinoline in an acute viral infection may be beneficial in boosting the Th1 antiviral response but prolonged Th1 stimulation may lead to problems with bacterial infections. In addition, use of these drugs after a severe bacterial infection might be beneficial in shifting away from the dominant Th2 response so as to prevent the subsequent establishment of chronic virus infections. Thus, successful manipulation of the immune response by use of the imidazoquinolines or other similar drugs could benefit patients with many different infections or conditions. These drugs may provide an entirely new means of helping patients when compared to existing treatment methods.

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Therapeutic response of basal cell carcinoma to the immune response modifier imiquimod 5% cream

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Background: Basal cell carcinoma (BCC) responds to interferon therapy. Imiquimod is a cytokine and interferon inducer.

Objective: This randomized, double-blind pilot trial evaluated the safety and efficacy of imiquimod 5% cream versus vehicle in the treatment of BCC.

Methods: In this population of 35 patients with BCC, 24 received imiquimod 5% cream and 11 received vehicle cream in 1 of 5 dosing regimens for up to 16 weeks. Six weeks after treatment, an excisional biopsy of the target site was performed.

Results: BCC cleared (on the basis of histologic examination) in all 15 patients (100%) dosed twice daily, once daily, and 3 times weekly; in 3 of 5 (60%) patients dosed twice weekly; 2 of 4 (50%) dosed once weekly; and in 1 of 11 (9%) treated with vehicle. Adverse events were predominantly local reactions at the target tumor site, with the incidence and severity of local skin reactions declining in groups dosed less frequently.

Conclusion: Imiquimod 5% cream shows clinical efficacy in the treatment of BCC. (J Am Acad Dermatol 1999;41:1002-7.)

Basal cell carcinoma (BCC) is the most common cutaneous malignancy.[1] Estimates of incidence of these tumors in the United States alone approach 1 million each year.[1-3] The current treatment of BCC is predominantly surgical. Simple excision is most commonly used, although curettage and electrodesiccation, cryosurgery, Mohs micrographic surgery, and laser surgery may be preferred in some cases.[1] Although the surgical modalities have a high cure rate and acceptable associated morbidity, a topically effective medical approach to the treatment of BCC may be of interest to some patients and practitioners.

Imiquimod is an immune response modifier that has been demonstrated to induce cytokines that promote a T_H1 or cell-mediated immune response.[4-6] These include interferon alfa (IFN-α), IFN-γ, and interleukin 12 (IL-12).[4,5] Induction of cytokines can be seen after both systemic and topical application.[5,6] In animal studies, imiquimod has demonstrated broad antiviral and antitumor effects that are largely mediated by IFN-α.[5] In humans, imiquimod 5% cream (Aldara, 3M Pharmaceuticals, St Paul, Minn) has been demonstrated to be safe and effective in the treatment of external anogenital warts,[7-9] with wart clearance associated with evidence of an increased T_H1 immune response and a reduction in human papillomavirus DNA.[10]

Because BCC is known to respond to IFN[11-13] and because imiquimod is a biologically active immune response modifier and IFN inducer, the present study was conducted to evaluate the safety and efficacy of imiquimod 5% cream in the treatment of BCC.

METHODS

Patients and eligibility

All patients signed an informed consent agreement, approved by an Institutional Review Board. Patients were eligible to participate if they had a biopsy-confirmed BCC

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Table I. Histologic type and anatomic location of target tumors by dose group

	No. (%) of patients by dose group					
	Imiquimod 5% cream dose groups					Vehicle
	Twice/day (n = 7)	Once/day (n = 4)	Three times/week (n = 4)	Twice/week (n = 5)	Once/week (n = 4)	(n = 11)
Histologic type						
Nodular	1 (14%)	1 (25%)	0	2 (40%)	2 (50%)	1 (9%)
Superficial	6 (86%)	3 (75%)	4 (100%)	3 (60%)	2 (50%)	10 (91%)
Anatomic location						
Upper extremity	4 (57%)	2 (50%)	1 (25%)	0	0	5 (46%)
Trunk: Anterior upper	1 (14%)	1 (25%)	1 (25%)	2 (40%)	1 (25%)	1 (9%)
Neck	2 (29%)	0	1 (25%)	1 (20%)	0	1 (9%)
Trunk: Posterior upper	0	1 (25%)	1 (25%)	1 (20%)	1 (25%)	0
Lower extremity	0	0	0	1 (20%)	2 (50%)	0
Trunk: Posterior lower	0	0	0	0	0	3 (27%)
Face: other	0	0	0	0	0	1 (9%)

with clearly visible margins that was nodular with an area of 0.5 to 1.5 cm², or superficial with an area of 0.5 to 2 cm², and that was suitable for treatment by surgical excision.

Randomization and treatment

Patients were unevenly randomized in a 2:1 ratio to receive imiquimod cream or vehicle cream. There were 5 treatment schedules: twice daily, once daily, three times weekly, twice weekly, and once weekly. Patients continued treatment with study cream until either 2 weeks after the target tumor was clinically cleared as determined by the investigator, or until 16 weeks of treatment were completed. Rest periods of up to 7 days were allowed if a patient was unable to comply with the dosing regimen because of local skin reactions or adverse events. Six weeks after completion of treatment the entire tumor site was surgically excised and thoroughly histologically examined by step sections for evidence of residual tumor. A patient was considered a complete responder if there was no histologic evidence of cancer in the excisional specimen.

Histologic processing

Elliptical specimens were bread-loafed from tip to tip into 2- to 4-mm thick slices, which were paraffin-embedded and step-sectioned at least every millimeter until the blocks were exhausted. In this way, the bulk of the tissue was examined histologically for evidence of residual tumor.

Safety evaluations

At clinic visits every 2 weeks, an investigator noted signs of local reactions. Local skin reactions were defined as erythema, edema, induration, vesicles, erosion, ulceration, excoriation/flaking, and scabbing. Signs and symptoms were scored on a 4-point scale where 0 = none, 1 = mild (visible local skin reaction without discomfort or with minimal discomfort but not disruption of normal daily activity), 2 = moderate (caused considerable discomfort but did not disrupt normal daily activities), and 3 = severe (substantially interfered with patient's normal daily activities). Adverse events reported by patients were also recorded at each visit.

Statistical analysis

As a pilot trial with a small number of patients, this study was not powered to detect a clinically meaningful difference between imiquimod and vehicle cream in the treatment of BCC; however, the complete response rate of BCC was estimated. The data set analyzed is the intent-to-treat data set, consisting of all randomized patients. Vehicle data from all the dose groups were combined to estimate vehicle effects.

RESULTS

Study patients

Of the 35 patients enrolled, 24 were randomized to receive imiquimod 5% cream (7 twice daily, 4 once daily, 4 three times weekly, 5 twice weekly, and 4 once weekly), and 11 patients were randomized to receive vehicle cream (3 twice daily, 2 once daily, 2 three times weekly, 2 twice weekly, and 2 once weekly). All study subjects were white, ranging in age from 37 to 81 years. Seven of the 35 patients had target tumors characterized as nodular; the remaining 28 patients had superficial tumors. Size of target tumors ranged from 0.5 cm² to 2.0 cm². Locations of target tumors were primarily on the upper body (Table I).

Efficacy analysis

Of the 24 patients who received imiquimod cream, 20 (83%) had complete histologic clearing of their target tumors with no evidence of BCC at the post treatment biopsy of the site. All of the patients

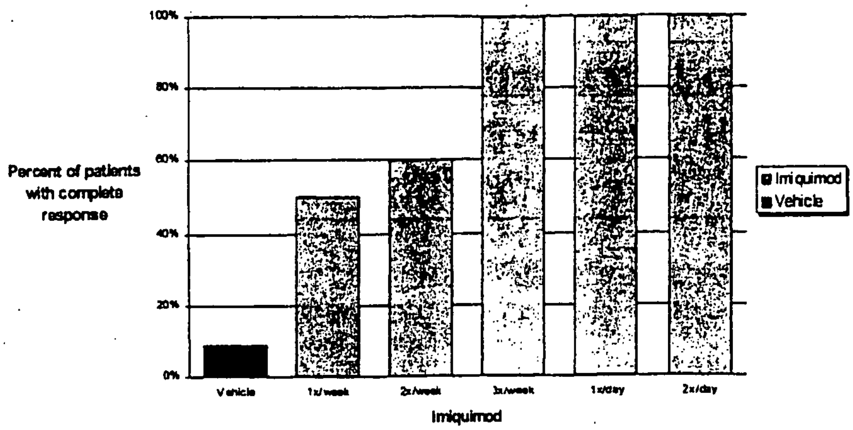

Fig 1. Dose/frequency response to imiquimod 5% cream and vehicle cream by treatment group.

Table II. Percent of patients with complete clearance of treated basal cell carcinoma and median length of treatment

Dose groups and treatment regimens	Complete responders	Median weeks of treatment
Imiquimod 5% cream		
Twice daily	100% (7/7)	10
Once daily	100% (4/4)	13
Three times weekly	100% (4/4)	14.5
Twice weekly	60% (3/5)	16
Once weekly	50% (2/4)	16
Vehicle cream	9% (1/11)	16

who received imiquimod cream under dosing regimens of twice daily (7/7), once daily (4/4), and 3 times weekly (4/4) experienced complete clearing of their BCC target tumors. With the twice-weekly and once-weekly dosing regimen, 3 of 5 (60%) and 2 of 4 (50%) patients, respectively, had complete clearing of their target tumors. Of the 11 patients receiving vehicle, only 1 (9%) patient had complete clearing (Fig 1). The median length of treatment for complete responders by dose group and weeks of treatment ranged from 10 to 16 weeks (Table II).

Safety analysis

Adverse events. All 35 (100%) patients enrolled reported at least 1 adverse event during the course of this trial. Application site reactions were reported by 22 of the 24 (92%) patients who were treated with imiquimod cream and by 7 of 11 (64%) patients treated with vehicle cream.

The most frequently reported application site reactions included itching at the target site, erythema at a remote site, discharge at the target site, papular rash at a remote site, and soreness, tenderness, and hypopigmentation at the target site (Table III). Most application site reactions were mild or moderate in intensity and generally well-tolerated by patients. Some patients took rest periods, more commonly in dose groups where imiquimod was applied more frequently. Rest periods were required by all of the twice daily, half of the once daily, and a quarter of the 3 times weekly subjects but by none of the other groups (Table III). The most frequently reported subjective systemic adverse events were fatigue, headache, fever, malaise, pain, nausea, diarrhea, and arthralgia (Table III). Adverse events of fever were based on subjective reports of fever or feeling feverish reported by patients between visits. Temperatures were measured at each visit and no fevers were noted.

Local skin reactions. Severe local reactions were observed by the investigator only in the twice-daily and once-daily groups. Both erythema and crusting were seen in 4 of 7 (57%) and 1 of 4 (25%) patients in the twice-daily and once-daily dose groups, respectively. Severe erosion was seen in 2 of 7 (29%) and 1 of 4 (25%) patients in the twice-daily and once-daily dose groups, respectively. Severe induration was observed in 1 of 4 (25%) patients in the once-daily group whereas severe ulceration was reported in 1 of 7 (14%) patients in the twice-daily dose group.

DISCUSSION

The response of BCC to imiquimod noted in this pilot study appears to be excellent. The response rates noted in twice-daily, once-daily, and 3 times weekly dose groups are clearly comparable to those achieved with surgical modalities. These findings

Table III. Application site reactions, rest periods, and systemic adverse events

	No. (%) of patients					
	Imiquimod 5% cream dose groups					
	Twice/day (n = 7)	Once/day (n = 4)	Three/week (n = 4)	Twice/week (n = 5)	Once/week (n = 4)	Vehicle (n = 11)
Application site reaction						
Itching at target site	6 (86%)	1 (25%)	2 (50%)	2 (40%)	1 (25%)	1 (9%)
Erythema at remote site	3 (43%)	1 (25%)	2 (50%)	3 (60%)	1 (25%)	0
Discharge at target site	5 (71%)	2 (50%)	1 (25%)	0	0	0
Papular rash at remote site	1 (14%)	1 (25%)	1 (25%)	2 (40%)	0	3 (27%)
Hypopigmentation at target site	0	2 (50%)	0	1 (20%)	0	2 (18%)
Soreness at target site	1 (14%)	2 (50%)	1 (25%)	0	0	1 (9%)
Tenderness at target site	3 (43%)	2 (50%)	0	0	0	0
Bleeding at target site	2 (29%)	0	0	0	0	2 (18%)
Sensitivity at target site	1 (14%)	0	0	0	0	2 (18%)
Burning at target site	1 (14%)	0	0	1 (20%)	0	0
Inflammation at remote site	0	0	1 (25%)	0	0	1 (9%)
Lesion at remote site	0	1 (25%)	0	1 (20%)	0	0
Necrosis at target site	1 (14%)	1 (25%)	0	0	0	0
Serous drainage at target site	1 (14%)	1 (25%)	0	0	0	0
Crusting at remote site	1 (14%)	0	1 (25%)	0	0	0
Rest periods						
No. of patients with rest periods	7 (100%)	2 (50%)	1 (25%)	0	0	0
Systemic adverse events						
Fatigue	1 (14%)	2 (50%)	0	0	0	0
Fever	2 (29%)	0	0	0	0	1 (9%)
Malaise	2 (29%)	1 (25%)	0	0	1 (25%)	1 (9%)
Pain	3 (43%)	1 (25%)	0	2 (40%)	1 (25%)	1 (9%)
Nausea	4 (57%)	2 (50%)	0	1 (20%)	0	0
Diarrhea	1 (14%)	0	0	0	0	1 (9%)
Arthralgia	1 (14%)	0	0	1 (20%)	1 (25%)	1 (9%)
Headache	2 (29%)	1 (25%)	3 (75%)	2 (40%)	3 (75%)	3 (27%)

raise a number of questions that relate to the cause of skin cancer and future approaches to skin cancer therapy.

There is a clear epidemiologic association between the susceptibility of individuals who sunburn easily with exposure to UV radiation and the onset of BCC. Traditionally, this is thought to result from direct DNA damage by the UV light radiation. UV radiation is also immunosuppressive to the skin.[3] The clinical response noted in this study of BCC to imiquimod would add additional evidence to the concept that cutaneous immunosuppression is involved in the onset of skin cancer. By immunosuppressing the epidermis, UV radiation prevents normal immune surveillance and allows the development of tumors from UV damaged keratinocytes. The current evidence suggesting an immunologic involvement in the occurrence of BCC includes (1) known immunosuppressive activity of UV radiation,[14,15] (2) an increased number of BCCs in renal allograft recipients,[16] (3) response of BCC to IFN,[11-13] and (4) the currently reported response of these tumors to imiquimod.

For UV light to produce an immunosuppressive effect, a chromophore is required to absorb the UV radiation, resulting in a signal that produces immunosuppression. DNA and *cis*-urocanic acid are candidates for immunosuppression chromophores.[17,18] *trans*-Urocanic acid is a normal constituent of the epidermis.[14] UV radiation results in the isomerization of *trans*- to *cis*-urocanic acid, which has many immunosuppressive effects.[14,17,19] *cis*-Urocanic acid can impair Langerhans cell antigen–presenting function of tumor-associated antigens in both primary and secondary immune responses.[17] Imiquimod has been demonstrated to increase the activity of antigen-presenting cells (personal correspondence: Mark Tomai, Oct 6, 1998). The exact mechanism of UV immunosuppression is not clear. Recently, the *cis*- to *trans*- transformation of urocanic acid by UV radiation has been implicated in UV-induced immunosuppression. Interestingly, the same cytokines that are down-regulated by urocanic acid are up-regulated by imiquimod.

It has recently been demonstrated that cutaneous basal cell and squamous cell carcinomas express T_H2 cytokines, specifically IL-4 and IL-10.[20] This observation has led to the hypothesis that it is IL-10 produced in the immunosuppressed epidermis that suppresses antitumor T cells. With successful treatment of BCC with interferon, there is an up-regulation of IL-2 and a down-regulation of IL-10 messenger RNA within the tumors.[20] Thus cytokine balance may be important in responses of BCC to immune response modifiers.

BCC can be divided histologically into aggressive and nonaggressive growth patterns. The nonaggressive growth patterns include superficial and nodular BCC that were the subject of this study. We have had no experience to date with imiquimod cream in treating the aggressive histologic types: namely morpheaform, sclerosing, or micronodular BCC. The latter types of BCC are traditionally more difficult to cure surgically and may be more difficult to treat medically.[21]

The anatomic location of the tumor may also influence response to therapy or recurrence of signs and symptoms after treatment. Traditional high-risk sites are predominantly central facial/periorificial; these high-risk sites were excluded from the current study. The medical approach to the treatment of BCC would appear most appropriate for nonaggressive histologic types of BCC present on low-risk anatomic sites, particularly the trunk. The trunk and extremities often heal with surgical scars cosmetically inferior to those achieved on the face.

In the study, the end point for efficacy was absence of histologic evidence of BCC as determined by aggressive step sectioning of the excised treated area. Because this is a histologic rather than a clinical end point, it would be considered a surrogate marker of efficacy. The clinical end point would be the absence of tumor at the end of treatment or the lack of recurrence of signs and symptoms of BCC over time. The reappearance of signs and symptoms of BCC after treatment has traditionally been called a recurrence. Recurrence of BCC after excision with "clear margins" most likely represents sampling error inherent in the routine histologic processing of elliptical samples. We believe this nomenclature is somewhat of a misnomer because recurrence implies that the tumor was eradicated but then reappeared. On a biologic basis, when the signs and symptoms of BCC recur after therapy they do so because there is persistence of tumor. It has been estimated that after incomplete surgical excision of BCC, 30% to 50% will recur.[21-24] If the tumor is totally removed, then it is not conceivable that the signs and symptoms would recur, and a significantly lower recurrence rate would be expected. It would seem logical that the persistence of tumor is associated with recurrence of signs and symptoms, and when aggressive histologic sectioning fails to reveal persistent tumor, one would conclude that the tumor has been adequately treated and the patient cured. The histologic step sectioning done in this study greatly exceeds the normal histologic examination of elliptical specimens in clinical practice.

The local inflammatory reactions noted in this study were acceptable in the treatment of a malignant condition. These brisk inflammatory reactions, at least clinically, would be consistent with an acute immunologic reconstitution of the sun-damaged skin resulting in an immunologically mediated elimination of malignant and premalignant cells. It should be noted that in phase I clinical testing, imiquimod 5% cream was no more irritating on normal skin than Vaseline Intensive Care Lotion (Chesebrough-Ponds, Greenwich, Conn) (personal correspondence: Dianna Dahl, Oct 6, 1998). This would suggest that the local inflammatory reactions are a reflection of cytokine induction in sun-damaged skin.

In addition to application site reactions, systemic adverse events were also reported. These included headache, fatigue, fever, malaise, pain, nausea, diarrhea, and arthralgia. There was a clustering of these events in the twice daily application group and they were sporadic in the other groups. The observed systemic adverse events are somewhat difficult to interpret in light of the small sample sizes. In previous large controlled trials in the treatment of genital warts,[8-10] there were no differences in the frequency or severity of systemic adverse events between imiquimod- and placebo-treated subjects. It is possible that these systemic reactions were related to therapy. Larger trials are needed to clarify the frequency and significance of these reactions.

The results of the present study are very promising for the development of a topically applied immunologically mediated medical treatment for BCC. This apparent efficacy needs to be confirmed in larger controlled trials before routine use of this modality can be recommended.

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Topical imiquimod treatment of a cutaneous melanoma metastasis

To the Editor: We read with interest the report of Beutner et al (J Am Acad Dermatol 1999;41:1002-7) demonstrating the efficacy of imiquimod (Aldara 5% Cream) for the treatment of basal cell carcinoma. We would like to add our own experience of the first successful treatment of cutaneous metastases of malignant melanoma by topical application of imiquimod. The immune response modifier imiquimod induces the release of the cytokines interferon alfa, interleukin 1β, interleukin 6, and tumor necrosis factor α by lymphocytes and other peripheral mononuclear blood cells.[1] In controlled trials using a 5% imiquimod cream for the treatment of anogenital viral warts, skin irritation was frequent, but no systemic toxicity has been observed.[2]

Based on the hypothesis that topical imiquimod may promote an immune response against melanoma cells, we treated a 50-year-old female patient suffering from disseminated cutaneous metastatic melanoma lesions. She presented in December 1998 with cutaneous metastases too widespread for excision or radiotherapy. Monochemotherapy was started with 850 mg dacarbazine/m^2 every 4 weeks. After 3 cycles, the initial lesions remained stable, but a new cluster of superficial cutaneous metastases emerged on the left breast (Fig 1, *A*). Histologic examination revealed melanoma cells, but no peritumoral inflammatory infiltrates. The largest lesion was 0.60 × 0.34 × 0.16 cm as measured by 7.5 MHz sonography. Topical treatment of these lesions

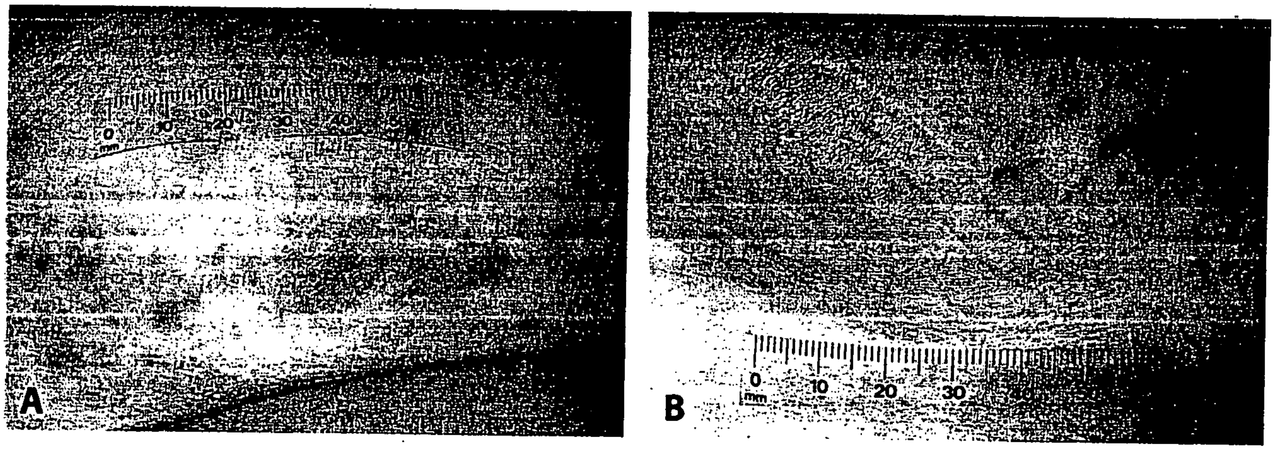

Fig 1. A, Melanoma metastases, left breast, before treatment (March 30, 1999). **B,** Residual hyperpigmentation and hypopigmentation and scars from biopsies, left breast, after treatment (Sept 7, 1999).

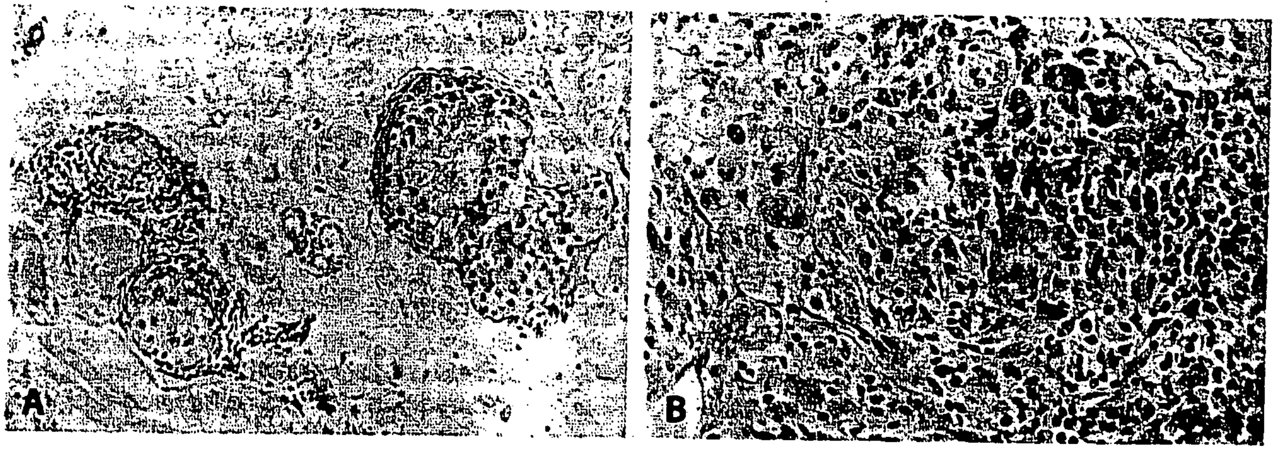

Fig 2. Photomicrographs of biopsy specimens after imiquimod treatment. **A,** Small tumor cell islands are surrounded by dense lymphocytic infiltrates. **B,** Dense lymphocytic infiltrates surround necrotic tumor cells, extensive presence of melanophages.

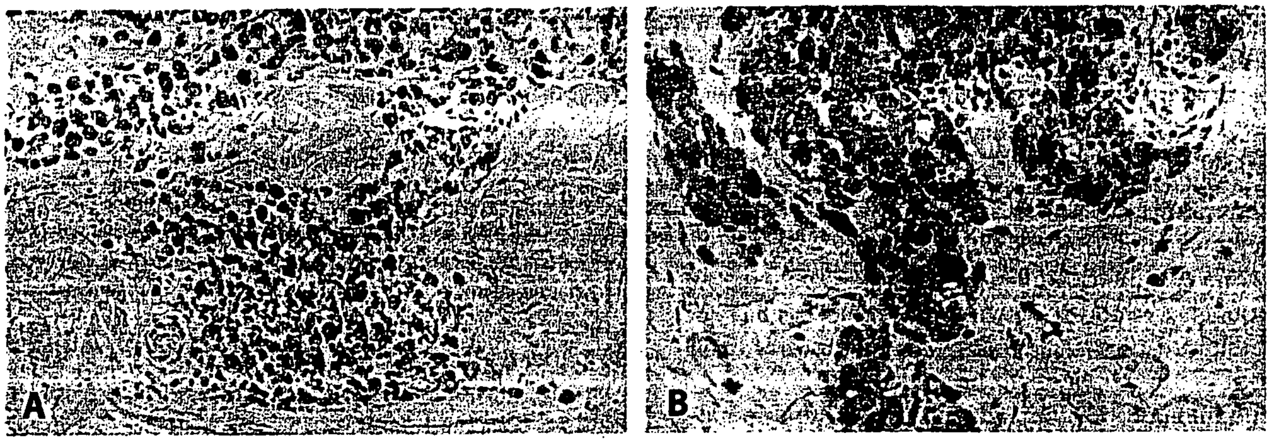

Fig 3. Photomicrographs of biopsy specimens after imiquimod treatment. **A,** Complete regression of tumor cells; only melanophages intermingled with lymphocytic infiltrates are present. **B,** Demonstration of CD8 expression on lymphocytes intermingling with melanophages.

with imiquimod was started 3 times weekly while dacarbazine therapy was continued. At the imiquimod-treated sites the tumor size decreased and 12 weeks later sonographic detection of these metastases was no longer possible (Fig 1, *B*). Simultaneously, untreated skin metastases progressed and new visceral metastases were diagnosed. Imiquimod therapy was continued for another 6 weeks, and another biopsy was performed on the marker lesion. The biopsy specimen showed apoptotic melanoma cells surrounded by dense lymphocytic infiltrates with a predominance of $CD8^+$ cells (Figs 2 and 3). A local irritation at the treatment sites, typical for imiquimod treatment, was the only side effect noted. Our observation suggests that a cellular immune response against the underlying cutaneous melanoma metastases was induced by topical imiquimod, which resulted in local control of tumor growth.

We believe that further investigation is warranted to determine whether imiquimod may be useful for the treatment of cutaneous melanoma metastases, especially when surgery or radiotherapy is not an option. We hypothesize that imiquimod induces local inflammation and "danger," resulting in apoptosis of tumor cells as well as maturation of dendritic cells.[3,4] This scenario may result in the induction of melanoma-specific cytotoxic T cells by cross-presentation of melanoma antigens by dendritic cells.[5]

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Wells syndrome associated with Churg-Strauss syndrome

To the Editor: Churg-Strauss syndrome (CSS), or allergic angiitis and granulomatosis, is a multisystem granulomatous vasculitis occurring in patients with asthma and characterized by eosinophilic infiltration of systemic organs and peripheral eosinophilia. Wells syndrome is a rare recurrent inflammatory dermatosis of indurated erythematous plaques with peripheral eosinophilia. The origin of Wells syndrome is unknown, but it has been reported in association with insect bites, parasites, viral infections, fungal infections, drugs, leukemic and myeloproliferative disorders, and atopic dermatitis.[1-3] Rarely, it has been associated with idiopathic hypereosinophilic syndrome, suggesting that there is an abnormal response of eosinophils.[4]

A 43-year-old woman had multiple grouped erythematous papules on the right palm and forearm

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Self-Administered Topical Imiquimod Treatment of Vulvar Intraepithelial Neoplasia

A Report of Four Cases

Gordon Davis, M.D., Jeffrey Wentworth, M.D., and Janet Richard, R.N.C., N.P.

BACKGROUND: Vulvar intraepithelial neoplasia (VIN) generally can be classified into viral and nonviral etiologies. The histopathologic diagnosis is often separable into basaloid and warty types. A large percentage of VIN lesions have been shown to harbor human papillomavirus (HPV), principally type 16. Imiquimod, an immune response modifier, has been shown to be safe and effective for the treatment of external and perianal genital warts caused by HPV.

CASES: Four cases occurred of clinical and histopathologically diagnosed viral VIN 3. An imiquimod treatment protocol, previously used in a study of this drug for the treatment of external genital warts, was followed. Imiquimod 5% cream was patient applied three times per week until all lesions cleared, for a maximum of 16 weeks.

CONCLUSION: Imiquimod may be an effective treatment modality for viral VIN 3 in the future. (J Reprod Med 2000;45:619–623)

Keywords: vulvar diseases; papillomavirus, human; imiquimod; vulvar intraepithelial neoplasia.

Imiquimod may be an effective treatment modality for viral VIN 3 in the future.

Introduction

Vulvar intraepithelial neoplasia (VIN) generally can be classified into viral and nonviral etiologies, each having different pathologic, virologic and clinical findings.[1-5] The histopathologic diagnosis is often separable into basaloid and warty types.[6] A large percentage of these lesions have been shown to harbor human papillomaviruses, principally type 16.[4,7] The incidence of viral VIN appears to be increasing and has almost tripled in white women under the age of 35, especially in cigarette smokers.[8]

Imiquimod is an immune response modifier that has been shown to be safe and effective for the treatment of external and perianal genital warts caused by human papillomavirus.[9-12] We present four cases of clinical viral VIN 3; we documented the disease by histology. An imiquimod treatment protocol (previously used in a study of this drug for the treatment of external genital warts) was followed. Imiquimod 5% cream was applied by the patient three days per week until all lesions cleared. The

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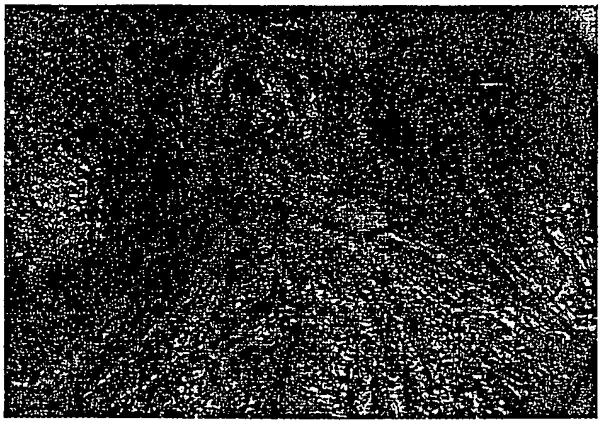

Figure 1 Case 1 before treatment. Multifocal, recurrent, warty VIN on both labia minora and the clitoral surface with associated subclinical HPV changes in the surrounding tissue.

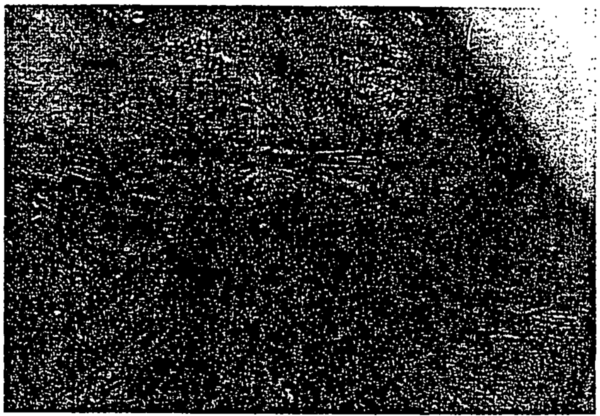

Figure 3 Case 2. Recurrent warty VIN 3 of the perianal skin prior to treatment.

maximum length of treatment offered was 16 weeks.

Case Reports

Case 1

A 32-year-old, Caucasian nonsmoker, G0, presented with a history of biopsy-proven warty VIN 3 that had recurred following partial vulvectomy and CO_2 laser ablation done three times. She presented with several recurrent vulvar lesions that were located in the periclitoral mucosa with extension to the distal glans clitoris (Figure 1). The vestibule had extensive papillary acetowhite changes, but the vagina, cervix and anus were free of disease colposcopically. Treatment with imiquimod 5% cream resulted in complete clearing of all lesions in one month (Figure 2). Posttreatment biopsy was negative.

At two months there was a small, recurrent warty lesion in the prior field of treatment at the junction of the right labium minus and clitoral frenulum. Imiquimod treatment was repeated. At three months, all lesions had cleared, and at one year there was no evidence of disease.

Case 2

A 35-year-old, Caucasian smoker with a history of recurrent VIN 3 had had treatment with topical

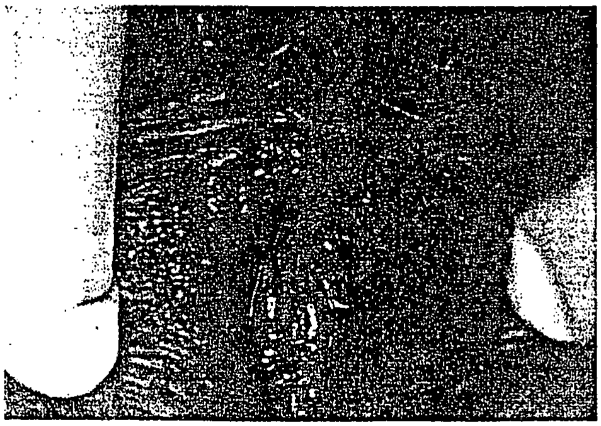

Figure 2 Case 1 after treatment with imiquimod for one month. Warty VIN 3 and subclinical HPV changes have cleared completely, and biopsy is negative. A small, warty VIN 3 recurred at two months but cleared with retreatment.

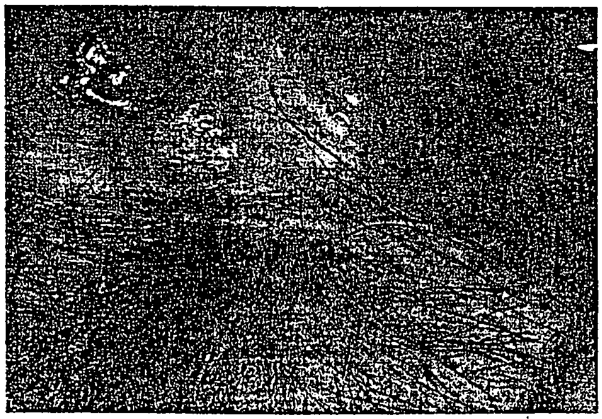

Figure 4 Case 2 after six weeks of treatment with imiquimod. VIN 3 has cleared, and histology is negative.

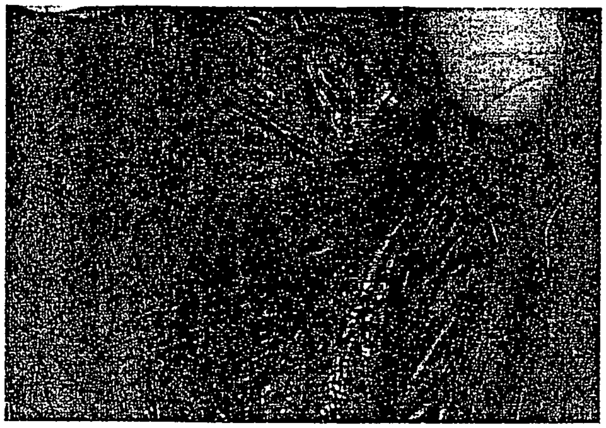

Figure 5 Case 3 before treatment. Multifocal, warty VIN 3 of right labium minus. The patient also had extensive vaginal HPV (VaIN) changes.

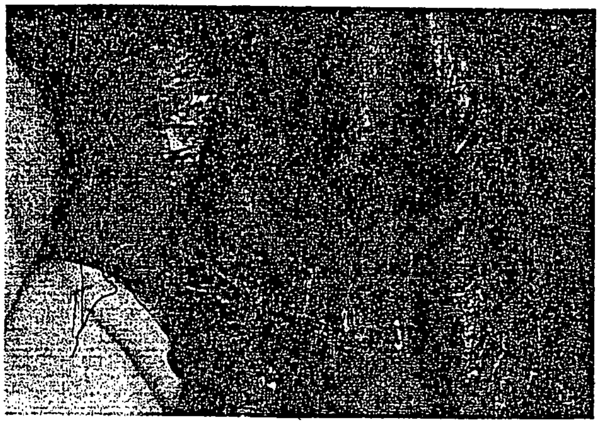

Figure 7 Case 4 before treatment, with erosive VIN 3 (basaloid histology) and warty VIN 3 on right labium minus (upper).

trichloroacetic acid, partial vulvectomy with vaginal advancement, repeat partial vulvectomy and CO_2 laser ablation of recurrent lesions. At routine follow-up following ablation, the patient complained of vulvar itching. Colposcopic examination revealed a 2-cm acetowhite region on the inner aspect of the right labium minus and warty, gray, acetowhite epithelium in the perianal region (Figure 3). Anoscopy with colposcopy was negative. Treatment with imiquimod 5% cream resulted in complete clearing at one, three and seven months (Figure 4). Posttreatment biopsy was negative. At one year perianal disease recurred.

Case 3

A 61-year-old, Caucasian nonsmoker had no known immunocompromise. Her past medical history was significant for interstitial cystitis and a total abdominal hysterectomy for endometriosis 30 years earlier. The patient presented with a complaint of vulvar itching and burning and having noted a lesion on the right labium minus. On physical examination, the right labium minus had a 3.5-cm, gray, micropapillary lesion (Figure 5). The skin of the upper perineum, contralateral labium and anus appeared to be free of disease. The vaginal vault contained extensive flat to micropapillary

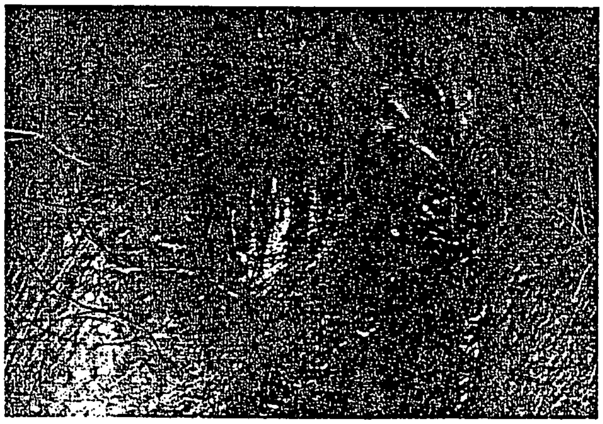

Figure 6 Case 3 after treatment. Multifocal, warty VIN 3 of right labium minus has cleared. The patient also had clearance of vaginal HPV (VaIN) changes.

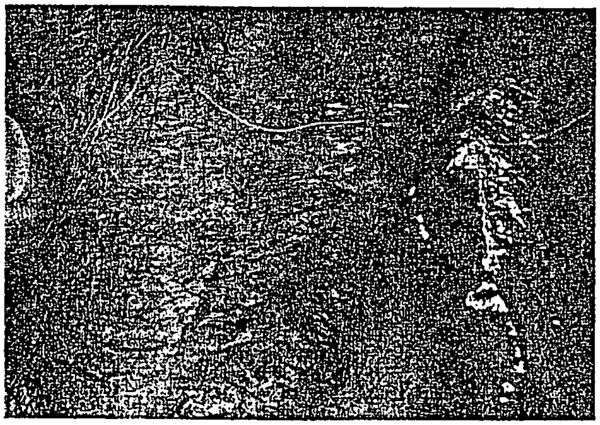

Figure 8 Case 4 after treatment. All VIN 3 on right labium minus has cleared. The basaloid VIN 3 recurred at six months and was retreated.

condylomata. Vulvar biopsy revealed VIN 3, and vaginal biopsy revealed vaginal intraepithelial neoplasia (VaIN) 1. The patient underwent imiquimod 5% cream treatment self-applied to the vulvar lesion three times per week. At the one-month follow-up, the vulvar lesion was smaller, and imiquimod therapy was continued. At the two-month interval, the vulvar lesion was much improved and appeared to be a pea-sized, keratinized area. Treatment was continued for one more month, during which the patient cleared the vulvar and vaginal lesions (Figure 6). At the three-month and one-year follow-up there were no colposcopically visible lesions. Posttreatment biopsy was negative.

Case 4

A 54-year-old, Caucasian nonsmoker had a history of Hodgkin's disease treated with chemotherapy in 1976. She had a long history of recurrent human papillomavirus (HPV) infection. Prior treatment included partial vulvectomy and laser surgery for VIN 3, vaginectomy for VaIN 3 and hysterectomy for cervical intraepithelial neoplasia 3. At the time of presentation, she had several new erosive red-gray lesions on the right labium minus associated with ipsilateral pigmented papules (Figure 7). Biopsy confirmed VIN 3, and anoscopy was negative. Treatment with imiquimod 5% cream resulted in complete clearing at the one-, two- and four-month follow-ups (Figure 8). A posttreatment biopsy was negative for VIN 3 in the treated region, but at one year the patient developed a recurrence outside the field of treatment.

Discussion

In 1989 the International Society for the Study of Vulvar Disease recommended a new classification system for vulvar disorders termed "nonneoplastic epithelial disorders of skin and mucosa."[13] This system separated such disorders as lichen sclerosus (nonneoplastic) from VIN (neoplastic). It now appears that VIN may be further classified into viral and nonviral etiologies. The typical patient with viral VIN is younger and HPV positive. Such patients often display multifocal lesions that tend to be warty and not associated with nonneoplastic diseases. It is clear that the treatment of viral and nonviral VIN could be different. The use of such treatments as partial vulvectomy and CO_2 laser ablation for viral VIN frequently results in recurrence, with estimates as high as 57%.[14] Most probably this high recurrence rate is secondary to multifocal disease and the presence of residual HPV after treatment.

In light of the suboptimal surgical treatment regimens, investigators have researched agents that could be effective for the treatment of VIN. Such agents as topical 5-fluorouracil, injected interferon, topical interferon and a combination of isotretinoin and interferon-alpha[15] have not proven successful. Vilmer et al[15] recently reported on combined therapy with isotretinoin and interferon-alpha, agents that have been reported to be active against HPV-related lesions. Their study of two cases resulted in apparent clinical clearance, but posttreatment biopsies showed histologic evidence of persistent VIN 3. Our treatment protocol included posttreatment biopsies of treated regions and did not show histopathologic evidence of persistent VIN 3 or HPV infection.

Of the four patients in our series, three presented with recurrent disease after surgery, and one had a new onset. One of the cases had a recurrence during imiquimod treatment; it was easily treated with reapplication. Another patient developed a late recurrence. The protocol entailed the use of imiquimod only until the lesions cleared; with extended treatment, as used in wart trials, this recurrence might not have developed.

Imiquimod may be an effective treatment modality for viral VIN 3 in the future. Further studies are needed for confirmation and to determine the length of therapy. Long-term follow-up of these and other patients is needed to answer the question of recurrent disease.

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THE CUTTING EDGE

SECTION EDITOR: GEORGE J. HRUZA, MD; ASSISTANT SECTION EDITORS: MICHAEL P. HEFFERNAN, MD; ELAINE SIEGFRIED, MD

Successful Treatment of Invasive Squamous Cell Carcinoma Using Topical Imiquimod

Ulrich R. Hengge, MD; Jörg Schaller, MD; Heinrich-Heine-University, Duesseldorf (Dr Hengge), and St Barbara Hospital, Duisburg (Dr Schaller), Germany

The Cutting Edge: Challenges in Medical and Surgical Therapeutics

REPORT OF A CASE

A 65-year-old man with a history of renal transplantation, chronic renal insufficiency after graft failure, and hemodialysis presented with a red palpable lesion on his right temple that had developed over a 3-year period (**Figure 1**A). The 4 × 3-cm erythematous hyperkeratotic plaque was located at the hair rim. On palpation, some induration and several papules were noted. Histologic examination of a 5-mm punch biopsy specimen showed tumor cells with variably irregular nuclei and several atypic mitoses that extended into the dermis (**Figure 2**A).

The patient's medical history was remarkable for metastatic prostate cancer and 5 years of hemodialysis because of chronic renal failure. Because of prostate cancer that had been diagnosed in 1998 and had metastasized to the seventh right rib, the patient was not undergoing chemotherapy. His most recent prostate-specific antigen level was within normal limits. His current medications were furosemide, simvastatin, and epoetin alfa.

THERAPEUTIC CHALLENGE

Our challenge was to use a noninvasive, conservative treatment in a patient with chronic renal failure and prostate cancer. Therapeutic modalities such as cryotherapy, excision, photodynamic therapy, and radiotherapy are associated with tissue destruction and/or substantial patient discomfort.[1,2] The recent success of topical immunomodulatory therapy with imiquimod for basal cell cancer,[3,4] actinic keratoses,[5] and intraepithelial carcinoma[6,7] prompted us to start the patient on a self-applied regimen of 5% imiquimod cream to be applied 3 times per week and left on overnight for 8 hours.

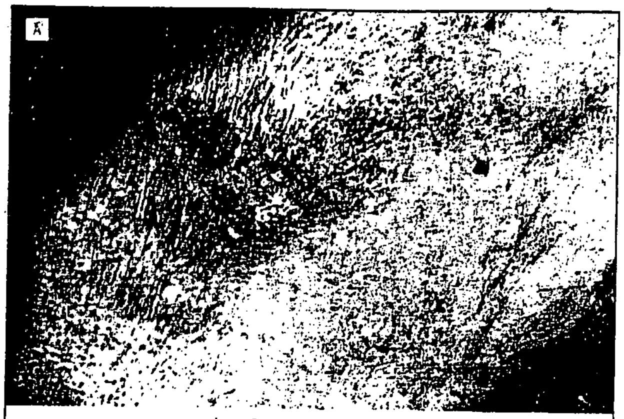
After 3 weeks of treatment, the lesion showed initial signs of regression at the borders, while the central erythema persisted (Figure 1B). The treatment was terminated at week 12. The patient reported no adverse effects other than some scaling. At week 16, he presented with a scar at the initial site of the lesion (Figure 1C), but there was no evidence of squamous cell carcinoma (SCC) in the biopsy specimens obtained from the borders and center of the area (Figure 2B).

COMMENT

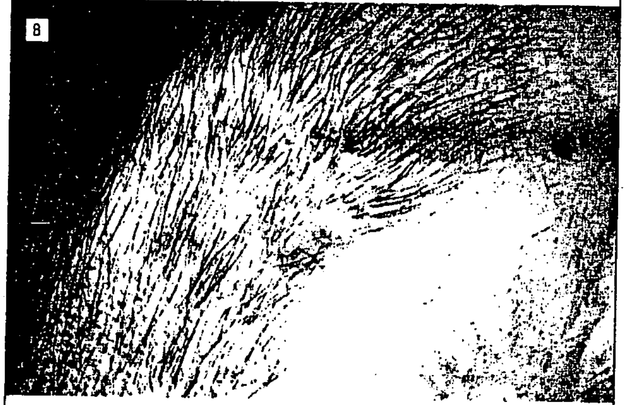
While several studies have shown the efficacy of imiquimod therapy for basal cell cancer,[3,4] in situ carcinoma (Morbus Bowen),[6-8] and actinic keratosis,[5] the present article reports the first case of invasive SCC successfully treated with topical imiquimod in a patient with chronic renal failure and prostate cancer. The histologic findings at the completion of treatment and the recurrence-free follow-up of 16 months suggest clinical cure.

Imiquimod belongs to a new class of topical immune response modifiers. It has been approved for the treatment of condylomata acuminata and has also shown efficacy in the treatment of other viral lesions, such as common warts, mollusca, and genital herpes.[9] The mechanism of action in humans is not completely understood, but it involves the stimulation of the cellular immune system after interaction with toll-like receptor 7, leading to the induction of several cytokines, such as interferon alfa, tumor necrosis factor α, and interleukin 12, from monocytes and macrophages.[9] Current thinking suggests that through the induction of interferon alfa, imiquimod may enhance antigen presentation by increasing the expression of mature histocompatibility class I and therefore, along with interleukin 12, augmenting the development of a type 1 helper T-cell immune response. Also, the maturation and migration of Langerhans cells may contribute to improved antigen processing and presentation.[10] In our patient, the 12-week treatment period was comparable to the length of imiquimod therapy needed to treat viral diseases.[11,12]

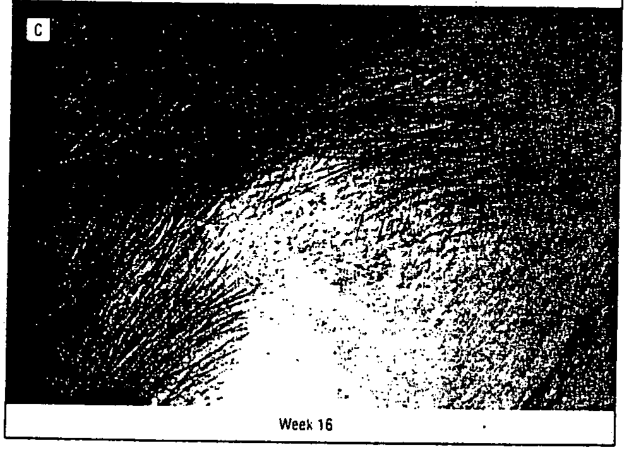
Since SCCs are not infrequently associated with human papillomavirus (HPV) in lesions in immunocompromised (84%) or immunocompetent patients,[13,14] a cell-mediated immune response against HPV seems possible.[9] Alternatively, cancerous antigens may serve as immunologic targets. In this regard, SCC antigens 1 and 2, which belong to the high-molecular-weight serine protease in-



Before Treatment



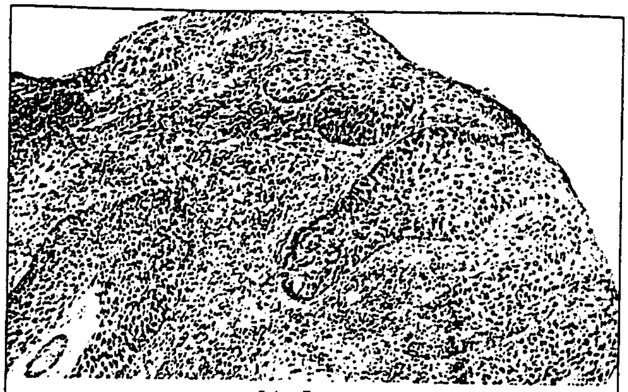
Week 4



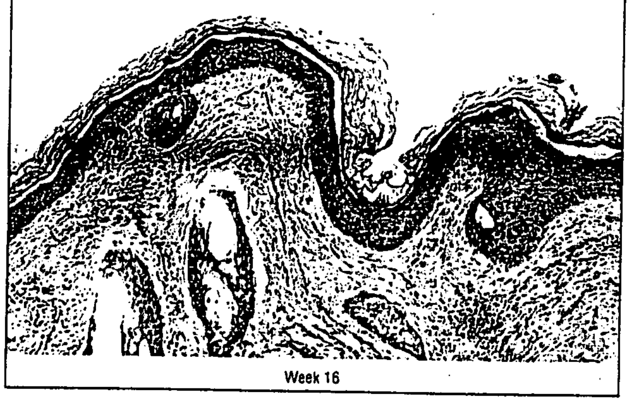
Week 16

Figure 1. A, Erythematous, hyperkeratotic plaque (4 × 3 cm) of 3 year's duration on the right temporal aspect of the hair rim. B, At week 4, initial regression is evident at the borders, while the central erythema persists. C, At week 16, a scar is seen at the lesion site.

hibitor (serpin) superfamily, may serve as tumor antigens.[15] Usually, SCC antigens 1 and 2 are coexpressed in the suprabasal layers of stratified squamous epithelium of the tongue, tonsils, esophagus, uterine cervix, and vagina.[15] However, they were recently detected in SCCs of the lungs and in cancers of the head and neck, where they were coexpressed in moderately to well-differentiated tumors, as in our case.[15] An alternative theory suggests that imiquimod directly induces apoptosis of tumor cells, as has recently been demonstrated in a study of basal cell carcinomas.[16] In that study, imiquimod was found to induce several mediators of apoptosis (eg, Fas, caspase 10, TRAF1, and TRADD) besides the up-regulation of interferon-inducible genes (eg, *MxA* and *MxB* and *STAT1* and *STAT2*), antigen-processing and presentation molecules (eg, PA28, TAP-1, PSMB6, and PSMB10), and immune-activation markers (CD40, CD86, LAG-3, RANTES, MIP-1R, and CCR7R).[16]



Before Treatment



Week 16

Figure 2. A, At presentation, histologic examination showed invasion of moderately differentiated tumor cells with variably irregular nuclei and atypic mitoses into the dermis (original magnification ×40). B, At the end of therapy, there was no evidence of squamous cell carcinoma, but increased fibrosis was observed (original magnification ×100). Specimens were formalin fixed and stained with hematoxylin-eosin. Subsequent sections were also stained with antikeratin antibodies and confirmed the absence of tumor cells in the dermis (data not shown).

The role of HPV in SCC in immunocompromised patients is still unclear. An extremely diverse group of HPV types, mainly consisting of epidermodysplasia verruciformis–associated HPV types, can be detected in benign, premalignant, and malignant skin lesions in organ transplant recipients.[14,15] Frequently, there are multiple HPV types present in single skin biopsy specimens. A comparison of transplant recipients with and without skin cancer, however, showed an equally high prevalence of epidermodysplasia verruciformis HPV DNA. The E6 protein from a range of cutaneous HPV types effectively inhibits apoptosis in response to UV-light–induced damage. It is therefore conceivable that individuals who are infected by epidermodysplasia verruciformis HPV are at an increased risk of developing SCC, possibly by chronically preventing UV-light–induced apoptosis in conjunction with their iatrogenic immunosuppression.

Our report also shows that topical immunomodulatory treatment is possible in severely compromised patients with renal failure and prostate cancer, indicating the intact quality of the skin-derived immune system under these conditions. However, the treatment should be reserved for selected patients and should be based on history of skin cancer, immune status, age, compliance, and reduced physical performance. While the potential for nonsurgical, patient-administered treatment of cutaneous malignant neoplasms in selected patients is great, extreme caution should be executed in clinical and histologic follow-up. Careful follow-up is strongly advised to detect lesions that are suggestive of recurrence. Histologic samples should be analyzed for response and margins. Furthermore, carefully designed studies are necessary to establish the usefulness of topical immunomodulatory therapy for SCC of the skin.

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The authors have no relevant financial interest in this article.

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Submissions

Clinicians, local and regional societies, residents, and fellows are invited to submit cases of challenges in management and therapeutics to this section. Cases should follow the established pattern. Submit 4 double-spaced copies of the manuscript with right margins nonjustified and 4 sets of the illustrations. Photomicrographs and illustrations must be clear and submitted as positive color transparencies (35-mm slides) or black-and-white prints. Do not submit color prints unless accompanied by original transparencies. Material should be accompanied by the required copyright transfer statement, as noted in "Instructions for Authors." Material for this section should be submitted to George J. Hruza, MD, Laser and Dermatologic Surgery Center Inc, 14377 Woodlake Dr, Suite 111, St Louis, MO 63017.

Clinical and Experimental Dermatology

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Front cover
The chemical structure of imiquimod.

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Clinical and Experimental Dermatology • **Concise report**

Topical treatment of intraepithelial penile carcinoma with imiquimod

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Summary

Intraepithelial penile carcinoma (IPC) is an *in situ* carcinoma of the penis, which can be difficult to diagnose. Current treatments include excisional surgery, Mohs' micrographic surgery, cryotherapy, carbon dioxide laser therapy and topical 5-fluorouracil. We report two cases of men with 12–18 month histories of IPC (Bowen's disease, squamous cell carcinoma *in situ*) that were previously unsuccessfully treated with antifungals and antibiotics. Treatment with imiquimod 5% cream for 8–10 weeks was effective in both cases with no clinical evidence of relapse at 4 and 6 months. Both patients experienced adverse effects, resulting in temporary discontinuation of treatment.

Introduction

Intraepithelial penile carcinoma (IPC) is an *in situ* carcinoma of the penis. Although the condition is clinically well known and defined, its diagnosis is often difficult. Identified risk factors that contribute to the development of penile cancer as a whole include cigarette smoking, sexual promiscuity and poor hygiene, but the strongest correlation exists with human papillomavirus (HPV).[1] HPV subtypes 16, 18, 31, 33 and 35 have been closely associated with cervical, anal, perianal, vulvar and penile carcinomas.[2–4] The rate of IPC has also been shown to be significantly higher in uncircumcised men.[5]

Due to the invasive potential of intraepithelial carcinomas, it is imperative that appropriate treatment is administered. Besides traditional excisional surgery, other treatment modalities include Mohs' micrographic surgery, cryotherapy, carbon dioxide laser therapy and topical 5-fluorouracil[6–8]. However, not all these methods are favoured, due to the associated physical and emotional scarring that may remain post-treatment, and the lack of histopathological confirmation of adequate clearing with treatments other than excisional or Mohs' surgery.

Imiquimod, a topically applied immune response modifier, with potent antiviral and antitumour activity *in vivo*, has demonstrated efficacy and has been approved for the treatment of external anogenital warts.[9,10] In addition, preliminary investigations have also shown its potential efficacy in the management of a number of other skin disorders.[11,12]

We describe the cases of two patients with IPC (non-HPV-related as confirmed by polymerase chain reaction), who underwent 8–10 weeks of treatment with imiquimod 5% cream followed by a 4–6 month follow-up.

Case studies

Patient 1

An otherwise healthy 56-year-old man presented with an 18-month history of IPC located on the glans around the uretheral meatus, measuring 8 cm^2. He had been previously unsuccessfully treated with a combination of topical antibiotics and antifungals. A 6-mm punch biopsy to evaluate histology of the lesion was performed (Fig. 1a). The lesion was treated with imiquimod 5% cream, applied three times a week for 6 weeks followed by a twice-weekly application over a 4-week period. The patient was compliant with this dosing regimen. However, during the course of treatment he experienced three episodes of severe erythema, accompanied with tingling, itching and pain. He also developed superficial

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erosion with intense oedema during week 5 of treatment. These local reactions resulted in temporary discontinuation of treatment. Three rest periods of 3–4 days each were taken, following which the treatment schedule was resumed. At 4-month follow-up, there was no clinical evidence of residual or recurrent tumour (Fig. 1b).

Patient 2

A 68-year-old diabetic man presented with IPC, with onset approximately 12 months earlier. The lesion located on the glans and extending to the coronary sulcus, measured approximately 3 cm^2 (Fig. 2a), and had been previously treated with a course of topical antibiotics and antifungals with no improvement. Pretreatment histological findings showed typical features of IPC. Treatment with imiquimod 5% cream was undertaken for a total duration of 8 weeks, during which the cream was applied three times weekly for the first 4 weeks followed by twice-weekly applications for the second 4 weeks. During the course of treatment, the patient experienced erythema, with associated burning and stinging, ranging from mild to moderate in intensity. Despite these local skin reactions, the patient was compliant with therapy, allowing for a few rest periods of 3–4 days each. During treatment the lesion reduced in total size (Fig. 2b), and showed both clinical (Fig. 2c) and histological resolution upon the completion of treatment. At the 6-month follow-up visit there was no clinical evidence of relapse.

Discussion

When left untreated, IPC may progress into invasive squamous cell carcinoma.[3,5] Curative therapy is therefore required. Traditional treatments have often proven unsatisfactory, due to associated recurrence, pain and scarring;[6–8,13] hence alternative means of therapy such as the application of imiquimod 5% cream need to be investigated. Imiquimod has previously been reported as successful for the treatment of *in situ* carcinomas of the penis.[12,14–16] It possesses both antiviral and antitumour activity *in vivo* that results from stimulation of innate and cell-mediated immunity.[17]

Both our patients with IPC were successfully treated with the application of imiquimod 5% cream. They reported local skin reactions, which were similar to

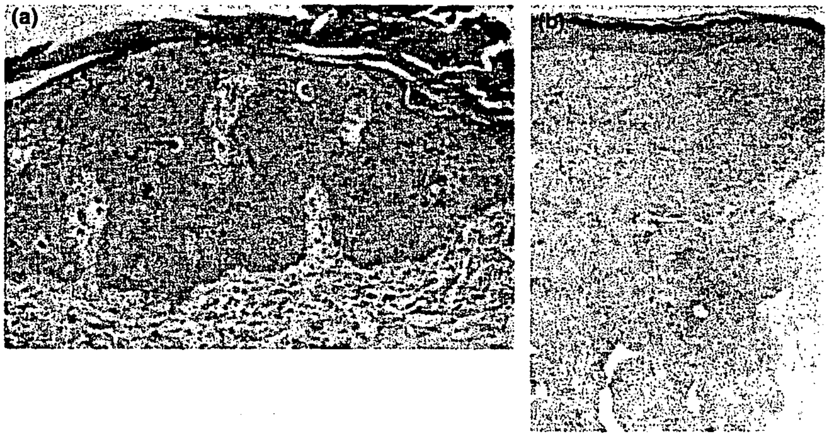

Figure 1 Histological findings (a) before and (b) after treatment with imiquimod 5% cream.

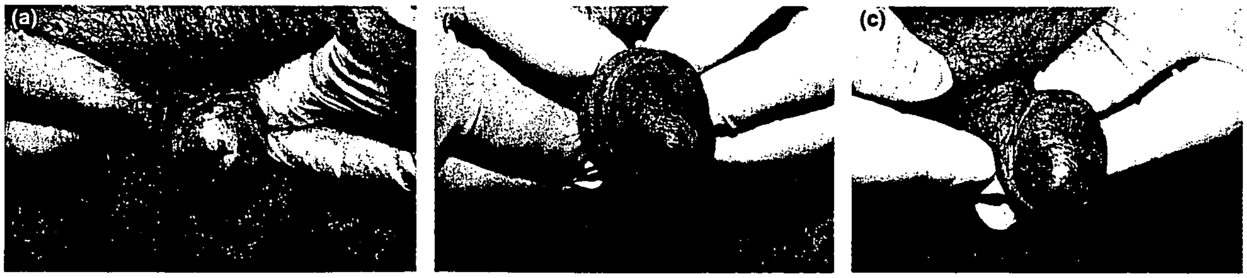

Figure 2 Intraepithelial penile carcinoma located on the glans penis of a 56-year-old man (a) prior to and (b) during treatment with imiquimod 5% cream. (c) Successful clearance at the end of treatment.

those previously observed.[18] These reactions were well tolerated, although they necessitated a brief rest period from the application of the cream.

The results in these two patients demonstrate several advantages of imiquimod. Firstly, imiquimod 5% cream may be an effective alternative form of treatment for IPC, and secondly, temporary discontinuation of treatment had no disruptive effect on overall therapeutic response. Finally, patient compliance and preference of this treatment compared with possibly disfiguring surgery, highlight the potential of imiquimod 5% cream for the treatment of IPC.

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Clinical and Experimental Dermatology • **Concise report**

Treatment of Bowen's disease of the penis with imiquimod 5% cream

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Summary

We present a case of persistent and progressive Bowen's disease (squamous cell carcinoma *in situ*) of the penis, in an otherwise healthy 56-year-old man. Treatment with imiquimod 5% cream was effective when applied once a day for 3 consecutive days followed by 4 days without treatment, over a period of 5 weeks.

Introduction

Bowen's disease (BD) is a form of intraepidermal squamous cell carcinoma *in situ*.[1,2] It is usually persistent and progressive, presenting as a gradually enlarging, well-demarcated, erythematous plaque with an irregular border and surface crusting or scaling.[1,2] Lesions tend to be solitary but can be multiple in 10–20% of patients.[1,2]

A small potential (approximately 3%) for invasive malignancy has been reported in several studies.[3,4] Treatment options vary with body site, and include cryotherapy, curettage, 5-fluorouracil, excision, laser and photodynamic therapy.[2,5] Spontaneous regression has also been observed.[3,4]

Genital lesions, which have the histology of BD, include erythroplasia of Queyrat and Bowenoid papulosis.[6,7] Erythroplasia of Queyrat (penile intraepithelial neoplasia) occurs on the glans penis and under the prepuce, virtually always in uncircumcised men. The risk of invasion for genital BD is higher (up to 10%) compared with other common sites of BD,[1,6] and treatment may need to be more aggressive. Surgery and destructive treatment modalities have a significant risk of scarring, deformity and impaired function. Recent case studies have reported the successful treatment of BD of the penis with topical imiquimod, an immune response modifier, as a 5% cream.[8,9] Cook-Bolden & Weinberg reported the first successful treatment of BD of the penis using imiquimod 5% cream daily for a total of 10 weeks.[8] There was no clinical evidence of recurrence 3 months after the post-treatment biopsy. Schroder & Sengelmann treated a BD lesion daily for a total of 24 days with a 2-week rest period, and there was no clinical evidence of recurrence 3 months after treatment.[9]

We present a case of BD of the penis treated with imiquimod 5% cream, daily for 3 consecutive days followed by 4 rest days, over a 5-week period.

Case report

A healthy 56-year-old man was referred with a persistent red lesion on the ventral part of his penis. It had arisen spontaneously about 15 years earlier and had enlarged over the years. The patient complained of erosion and weeping from the lesion. Before being referred to our clinic, the patient had received various different treatments (local steroids, local antibiotics) with limited effect.

At the examination visit, we observed a 1.5 × 1.5 cm firm, red and shiny eroded plaque with slightly elevated uneven borders extending proximal from the frenulum preputii (Fig. 1a). The differential diagnoses considered were BD and plasma cell balanitis. A biopsy was performed from the edge of the plaque, which confirmed the diagnosis of BD.

Treatment with imiquimod 5% cream once a day for 3 consecutive days followed by 4 days without any treatment was started. Hours after applying the cream, the patient experienced local irritation with redness and swelling, which occurred on only one occasion. At the same time the patient complained of influenza-like symptoms, with chills and leg pain. The patient did

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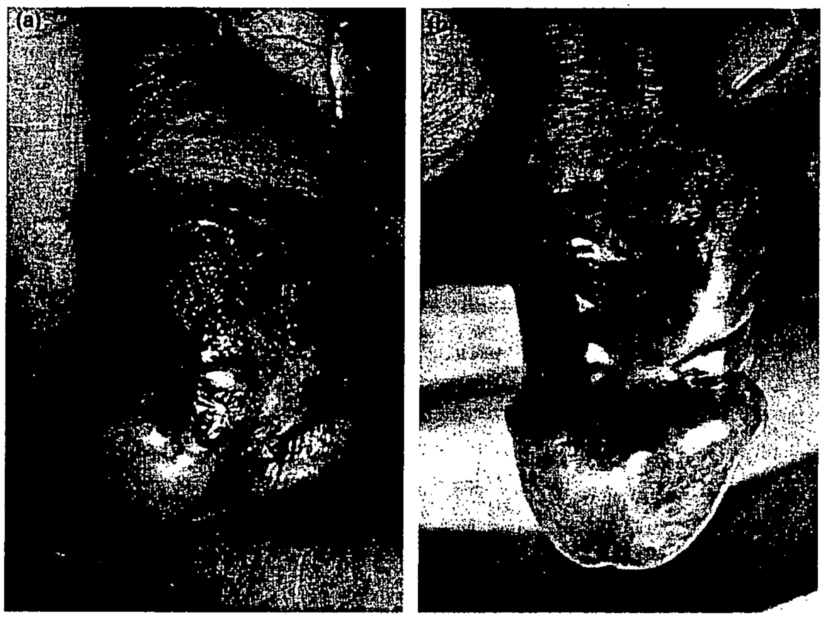

Figure 1 Bowen's disease of the penis treated with imiquimod 5% cream (a) before treatment, and (b) after five weeks of treatment.

not measure his temperature, did not think that he had a fever, and continued his work as usual. The flu-like symptoms resolved within approximately 12 h. Haemoglobin, thrombocyte and leucocyte counts were normal during the treatment. After a period of 5 weeks the lesion was partially cleared (Fig. 1b). Three months after the end of treatment there was no evidence of the lesion.

Discussion

Bowen's disease of the penis has previously been treated with variety of therapies;[8,10–12] however, the appropriate treatment option depends on site of the lesion. Cox and Morton have prepared guidelines on behalf of the British Association of Dermatologists for the management of BD.[2] They suggest that radiotherapy, 5FU, photodynamic therapy[10] and cryotherapy[11] are potentially useful to treat genital BD. They also note that Mohs' micrographic surgery has been used for genital BD but only reported in two cases.[12] Interferon-α and -γ have both been used systemically or interlesionally as a treatment for BD induced by human papillomavirus in the genital and perianal area.[13,14] This case report suggests imiquimod 5% cream as an alternative to these therapies in the treatment of penile BD.

The local skin reaction observed in this case was also evident in a Phase II open-label study of 16 patients with BD treated with imiquimod therapy.[15] Mackenzie-Wood *et al.* noted that in the majority of patients an increased local skin reaction was present at the 1-week review. They found that patients ($n = 6$) who discontinued treatment due to an exaggerated reaction at the treatment site had improved within 2 weeks of ceasing imiquimod.[15] This has also been demonstrated with other applications of imiquimod, such as in the treatment of BCC,[16–19] and also in this study, as despite the patient discontinuing the treatment regimen early, the subsequent biopsy 6 weeks later showed no residual BD.

The flu-like symptoms this patient experienced did not lead to discontinuation of the therapy. No other medication was taken by the patient, and although he was aware of his symptoms, they did not bother him a great deal. We propose that imiquimod 5% cream may provide an alternative for the treatment of penile BD and we continue to monitor our patient to confirm long-term clearance.

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Clinical and Experimental Dermatology • **Concise report**

Treatment of Bowen's disease using a cycle regimen of imiquimod 5% cream

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Summary

Bowen's disease (BD; intraepithelial squamous cell carcinoma) is a challenging condition to treat because lesions, which can be multiple, are often located at sites that heal poorly, such as the shin. The disease is usually persistent and progressive and appears as an enlarging, demarcated erythematous plaque. Two elderly female patients with Bowen's disease of the lower leg are presented. Imiquimod 5% cream was applied in a cycle of three times weekly for 3 weeks followed by a 4-week rest period. The treatment was successful after a second cycle of therapy, with both cases clinically clear at 2- and 3-month follow-up visits.

Introduction

Bowen's disease (BD) is a form of intraepidermal (*in situ*) squamous cell carcinoma (SCC) that appears as a slowly enlarging, sharply demarcated erythematous plaque. It is usually persistent and progressive with potential for invasive malignancies, although spontaneous partial regression may occur. Exposure to sunlight, human papillomavirus, radiation therapy and arsenic ingestion have been implicated in the pathogenesis of BD. Treatment modalities include surgery, curettage and cautery, cryotherapy, 5-fluorouracil, laser therapy, radiotherapy, photodynamic therapy or local injections of interferon-α or -γ.[1,2] Even with this wide range of treatment modalities, there is not always a feasible treatment option for large lesions on anatomically difficult areas such as the shins. Furthermore, elderly patients with poor wound healing and comorbidities may not tolerate these procedures.

Imiquimod is a topical immune response modifier, shown to have indirect antiviral and antitumour effects through the stimulation of local cytokine production and cell-mediated immune response.[3,4] Recent research has demonstrated its effective usage as a 5% cream in the management of BD.[2,5]

We report two patients with BD of the lower limbs successfully treated with imiquimod 5% cream using a cycle regimen. Each cycle comprised three times weekly applications for 3 weeks, followed by a rest period of 4 weeks.

Case studies

Patient 1

An 86-year-old white female with Fitzpatrick type II skin presented with an irregular scaly erythematous plaque over her left shin approximately 8 cm at its maximum diameter (Fig. 1a). She was otherwise well with no significant medical conditions. Skin biopsy confirmed BD with full-thickness epidermal dysplasia and parakeratosis.

She was instructed to apply imiquimod 5% cream topically three times weekly for 3 weeks on an area approximating 10 × 7 cm, followed by a rest period of 4 weeks. At the end of this first treatment cycle, the lesion had improved but was not clinically clear (Fig. 1b). She therefore underwent a second cycle of therapy. At the end of this cycle, the lesion was clinically clear (Fig. 1c).

Repeat biopsy at the end of the second cycle showed complete resolution of the BD with a chronic inflammatory infiltrate noted in the dermis. She was reviewed

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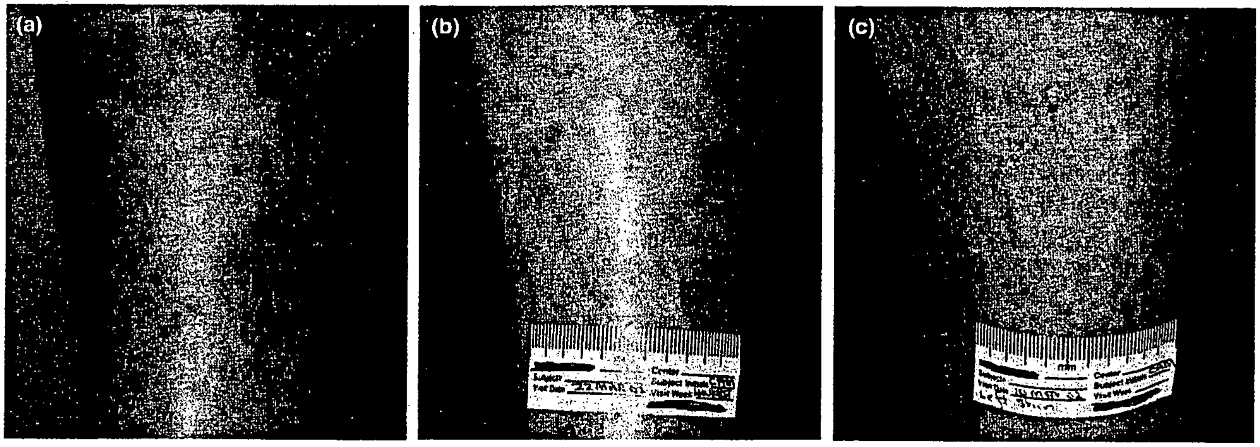

Figure 1 (a) Irregular area of Bowen's disease over left shin at start of therapy; (b) clinical improvement at end of first treatment cycle with imiquimod; (c) clinical resolution at end of second treatment cycle with imiquimod.

regularly during the treatment periods and no significant local skin reactions (e.g. erythema) or adverse events were noted. At the 2-month follow-up visit, she remained clinically clear.

Patient 2

A 64-year-old white female with Fitzpatrick type I skin and a long history of actinic damage presented with a scaly plaque on her left leg. Her previous treatment history included the removal of multiple skin tumours including excision of a small patch of BD on her lower left leg the previous year. Her medical background included a history of hypercholesterolaemia. On examination, she had a scaly plaque approximately 2 cm in diameter on her left shin, away from the site of previous BD. Skin biopsy confirmed the diagnosis of BD. The surrounding skin showed severe actinic damage, cellular atypia and hyperkeratosis consistent with BD.

She was instructed to apply imiquimod 5% cream topically three times weekly for 3 weeks onto an area approximately 12 × 10 cm, encompassing the Bowen's lesion. At the end of the first 3 weeks of treatment, the patient had a 4-week rest period, after which the treatment area showed improvement, but clinical evidence of residual BD remained. This resulted in her commencing a second treatment cycle. Following two completed treatment cycles, a repeat biopsy showed mild residual BD with mild nuclear atypia, occasional mitoses and slightly atypical keratinocytes. A lymphocytic infiltrate was noted in the dermis. Another biopsy 2 weeks later showed complete resolution. Treatment with imiquimod 5% cream was well-tolerated with no severe local skin reactions noted. At the 3-month follow-up visit, the treatment site remained clinically clear.

Discussion

In both patients, the BD was located on an area where surgical procedures would have been difficult. Our results confirm previous findings on the effective therapeutic benefits of imiquimod 5% cream in the treatment of BD in areas such as the penis.[5,6] The availability of a locally effective, nonsurgical therapy such as imiquimod 5% cream is a potentially valuable alternative in the management of such patients.

Optimal management involves clearing the BD while producing minimal, well-tolerated local skin reactions. The severity of local skin reactions with imiquimod tend to be related to the dosing regimen. In a study involving daily use of imiquimod for 16 weeks for BD, 38% of subjects ceased treatment early due to local skin reactions.[2] However, most of these patients subsequently showed clear post-treatment biopsies, raising the possibility that less intensive treatment regimens may be effective. On the basis of this observation, we undertook a cycle regimen involving three times weekly application of imiquimod for 3 weeks, followed by a rest period of 4 weeks, to be repeated if necessary. The rest period allows any local skin reactions to subside. In addition, it has been proposed that there is a 'point of no return' when imiquimod stimulates the immune response.[7] At that point, treated lesions may be destined for destruction and therefore continue to improve after cessation of therapy. This is demonstrated in the second patient, where serial biopsies over 2 weeks showed resolution of the BD despite therapy having been discontinued 4 weeks previously. Continuing therapy after this 'point of no return' may thus produce more local skin reactions, but no added efficacy.

The two patients tolerated the regimen of three times weekly doses for 3 weeks with no significant local skin reactions noted, and resulted in complete resolution of the BD. In conclusion, cycle therapy with imiquimod 5% cream may therefore be an alternative treatment option for selected patients with BD.

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Clinical and Experimental Dermatology • **Concise report**

Treatment of large facial Bowen's disease: case report

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Summary

Bowen's disease (BD; squamous cell carcinoma *in situ*) is a common, persistent condition that can be related to chronic sun damage, and consequently, is usually located around the head and neck area and lower limbs. Bowen's disease can be treated with a variety of methods, including surgery or laser therapy, but large lesions tend to scar postexcision and hence are difficult to treat surgically. Here we present the case of a 75-year-old woman with a 20-year history of facial BD unabated by treatment with a variety of topical agents and cryotherapy. Application of imiquimod 5% cream on alternate nights for 6 weeks resulted in total clearance with no recurrence observed after 8 months.

Introduction

Bowen's disease (BD; squamous cell carcinoma *in situ*) is a common disease suggestive of a relationship with chronic solar damage,[1,2] especially considering the age group (over 60 years old) and body site distribution of BD (head and neck, female lower leg).[3,4] Lesions are usually solitary but can be multiple in 10–20% of patients.[3]

BD can be treated by surgery, curettage and cautery, cryotherapy, 5-fluorouracil, laser therapy, radiotherapy, or photodynamic therapy.[5] However, large skin cancers may be difficult to treat surgically because of the potential for scarring after removal.[6] An alternative to surgery is imiquimod, an immune response modifier, which is applied topically as a 5% cream. In an open-label phase II study, imiquimod 5% cream was found to be effective when used to treat BD; 16 patients with a single lesion on their lower leg were treated with imiquimod daily for 16 weeks.[5] However, Mackenzie-Wood *et al.* suggested that alternative treatment regimens (i.e. not daily) for imiquimod should be investigated in order to minimize local reactions.[5] Imiquimod therapy has also been used to successfully treat superficial basal cell carcinoma and actinic keratosis.[6–9]

We present a case of large facial BD present for over 20 years treated successfully with imiquimod 5% cream on alternate days, over a 6-week period.

Case report

A 75-year-old woman presented with a 20-year history of an enlarging red scaly patch over the right preauricular area and cheek (Fig. 1a). Over the years the patient had applied topical corticosteroid creams, moisturizers and sunscreens, but the red lesion failed to resolve. Part of the lesion had been previously treated by a dermatologist with cryotherapy using liquid nitrogen, which produced an area of hypopigmentation.

One month prior to consultation a biopsy had been obtained and the lesion was diagnosed as BD. The area measured 6 × 5 cm in diameter and had an erythematous and scaly surface with a patchy zone of hypopigmentation. Palpation did not reveal any infiltrated component or evidence of local lymphadenopathy.

The patient was instructed to apply imiquimod 5% cream on alternate nights to the lesion. There was a rapid local skin reaction (erythema) within the first week of treatment. At 4 weeks, the area was eroded and measured 9 × 8 cm in diameter and there was a peripheral haemorrhagic eschar (Fig. 1b). The patient was able to tolerate the cream despite the severe local skin reaction. Treatment was continued for a further 2 weeks, with a total length of treatment of 6 weeks. There was no clinical evidence of BD at the end of

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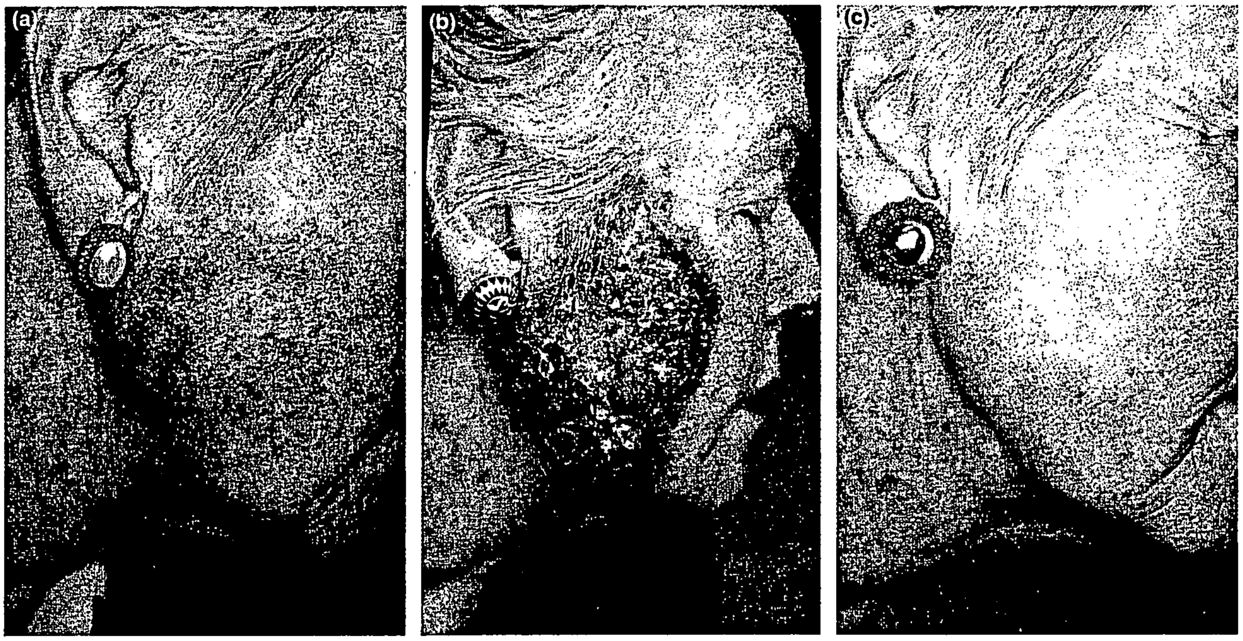

Figure 1 Large facial Bowen's disease treated with imiquimod cream: (a) before treatment; (b) during treatment, showing peripheral haemorrhagic eschar; (c) following treatment, showing area of hypopigmentation caused by previous cryotherapy.

treatment. The area of local skin reaction healed over a 3-week period following treatment.

At a follow-up visit, 8 months after completion of treatment, there was no residual areas of BD (Fig. 1c). There was a focus of hypopigmentation caused by the previous cryotherapy.

Discussion

Bowen's disease has previously been treated with a variety of therapies, for example surgery, cryotherapy, radiation therapy, and photodynamic therapy.[10] Ahmed *et al.* recently compared the efficacy of cryotherapy with curettage and cautery (C & C) in the treatment of BD. They found that during treatment and the subsequent 24 h, patients were 10 times more likely to report pain of any degree for lesions treated by cryotherapy than by C & C.[11] In the cryotherapy group ($n = 36$ lesions) the median time to heal was 46 days (range 14–210), with 13 (36%) of the treated lesions recurring by 24 months of follow up. In the C & C group ($n = 40$ lesions) the median time to heal was 35 days (range 14–330) with recurrence observed in four lesions over the follow-up period. Ahmed *et al.* suggested that C & C is superior over cryotherapy in the treatment of BD.[11] However, C & C is suboptimal for large lesions, and may be complicated by poor healing and obvious scars.

Photodynamic therapy has been demonstrated to be an effective tissue-sparing modality, achieving good cosmesis in patients with large or multiple BD.[12] Studies report a recurrence rate of 0–11% during 12 months' follow-up.[10] However, availability of photodynamic therapy is limited in some countries and the procedure can be time-consuming.

In this case, topical application of imiquimod 5% cream to the lesion on alternate nights for 6 weeks resulted in no clinical evidence of BD 8 months after treatment. The local skin reaction was probably due to an inflammatory response, which resolved once treatment was completed. The phase II open-label study also found that imiquimod can induce local skin reactions in most patients but it does not usually affect the normal surrounding skin.[5] Mackenzie-Wood *et al.* noted that six patients had their treatment ceased as early as 4–8 weeks because of severe local skin reactions; however, the site improved within 2 weeks.[5] The treatment response so far in this case study (no residual at 8 months' follow-up) indicates that imiquimod 5% cream can successfully treat large BD lesions, and that it may be possible to apply the cream over a shorter time period (6 weeks) than previously reported.

This reports only a single case of large BD treated with imiquimod, therefore further multicentre trials are needed to assess the efficacy of imiquimod 5% cream in the treatment of large BD. It is also important that the

BD lesion be carefully examined for infiltrated areas, as the efficacy of imiquimod in the presence of invasive squamous cell carcinoma has not been demonstrated.

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